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14. ABSTRACT

This study investigated the carcinogenic and immunotoxic potential of embedded fragments of depleted uranium (DU) and a heavy-metal tungsten alloy (WA) consisting of tungsten, nickel, and cobalt. Male Fisher 344 rats were surgically implanted with pellets of DU, WA, tantalum (inert metal, negative control), or nickel (known carcinogen, positive control). Implanted WA resulted in the rapid formation of tumors, identified as rhabdomyosarcomas, surrounding the pellets. These tumors had, within the same area, histopathological characteristics of both the pleomorphic and embryonal subtypes of rhabdomyosarcomas. Eventually these tumors metastasized to the lung. Rats implanted with tantalum or DU pellets did not develop tumors at the implantation site. In addition, WA-implanted rats (high-dose group) exhibited splenomegaly and hematological changes suggesting polycythemia as early as 1 month after pellet implantation.

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INTRODUCTION

Advancement in weapons design has led to the introduction of several potentially toxic metals, such as depleted uranium (DU), onto the battlefield. The Persian Gulf War in 1991 saw the first combat use of DU kinetic penetrator munitions, and their success against enemy armor was dramatic. The demonstrated effectiveness of DU munitions in the first Gulf War has led other nations, some not friendly to the United States, to adopt these weapons into their own arsenals. Other types of kinetic energy penetrators use heavy-metal tungsten alloys (WA) in place of DU. In future conflicts, the United States will have to deal with an increased number of casualties from the use of these weapons. Because both DU- and WA-based munitions are relatively recent additions to the list of militarily relevant metals, little is known about the health effects of these metals after internalization as embedded shrapnel. This study was designed to assess the carcinogenic and immunotoxic potential of DU and WA using the Fisher 344 rat model and modified National Toxicology Program protocols for such studies. Responses to the test metals were compared to responses to tantalum, a biologically inert metal that serves as a negative control and nickel, a known heavy-metal toxin and carcinogen that serves as a positive control. This research addresses the DOD effort to understand the potential health risks associated with DU and WA exposure in order to develop appropriate medical treatment protocols for personnel wounded by fragments of these metals.

BODY

Statement of Work (taken from the USAMRMC-approved proposal)

This study is an assessment of the immunotoxic and carcinogenic potential of embedded fragments of DU and WA in laboratory rats. Responses to these metals are compared to the biologically inert metal, tantalum, and the carcinogen and heavy-metal toxin, nickel. For these experiments, rats are implanted with tantalum pellets alone (metal control group), a mixture of DU and tantalum pellets (low DU group), DU pellets alone (high DU group), a mixture of WA and tantalum pellets (low WA group), or WA pellets alone (high WA group). There is also a non-surgical control group and a positive carcinogenesis control group implanted with nickel pellets. Animals will be euthanized and various analyses performed 1, 3, 6, 12, 18, and 24 months after implantation. Analyses include histopathological examination and metal determinations as well as assessments of mutagenicity and cytogenicity. A battery of immunological tests designed to assess both humoral and cell-mediated immunity, as well as the innate immune response, will be conducted at 1, 3, 6, and 12 months.

Progress to Date

This is the final report for this project. All five tasks associated with the project have been successfully completed. Research results for most of the task areas have been presented in detail in previous annual reports. However, as per USAMRMC requirements governing final reports, they are again presented below. There are two major sections for each task area. First, the experimental design for the task will be described. This will be followed by a detailed description of the results obtained for the task area. All data referred to in those sections can be found in the Appendices.

Experimental Approach

The Fischer 344 rat model was used for these experiments. The number and dimensions of the pellets (DU, WA, Ni, tantalum) were based on research previously conducted at AFRRI (Castro et al., 1996). This study was designed provide carcinogenicity, genotoxicity, and mutagenicity data for two DU doses, two WA doses (low and high), and one Ni dose (to serve as a positive control) at four times after pellet implantation (6, 12, 18, and 24 months). This cohort

would also provide immunotoxicity data for two DU doses and two WA doses (low and high) at four time points (1, 3, 6, and 12 months). Rats were randomly assigned to one of six treatment groups. These groups were: 1) rats implanted with low-dose DU, 2) rats implanted with high-dose DU, 3) rats implanted with low-dose WA, 4) rats implanted with high-dose WA, 5) rats implanted with high-dose nickel, 6) rats implanted with tantalum to control for fragment implantation, and 7) a non-implanted control group. In the low-dose DU and WA groups, tantalum pellets were also implanted so that the total number of implanted fragments in all animals was constant. At various times, rats were euthanized and assessments made for evidence of cancer (Task 1) and immunotoxicity (Task 4), and tissue metal content determined (Task 2). Using tissues from these animals, we also assessed genotoxicity and mutagenicity (Task 3), and changes in immune function (Task 5) resulting from chronic *in vivo* exposure to DU and WA.

General Methods

Subjects: Fischer 344 male rats were maintained in an AAALAC-accredited facility in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Research Council, 1996). Upon arrival, rats were quarantined and screened for diseases. Animals were housed in plastic microisolator rat cages with hardwood chips for bedding and fed a certified NTP-2000 diet to prevent excessive weight gain and to enhance longevity (Rao, 1996; Keenan et al., 1999). Acidified water (pH 2.5-2.8, using HCl) was provided *ad libitum*. Rats were on a 12-hr light/dark cycle, with no twilight (lights on at 0600) and were weighed weekly.

Fragments: DU fragments were obtained from Aerojet Ordnance Tennessee (Jonesborough, TN) and consisted of 99.25% DU and 0.75% titanium by weight. The uranium isotopes present were ^{238}U (99.75%), ^{235}U (0.20%), and ^{234}U (trace). This is the same DU alloy used in U.S. military munitions. Aerojet also provided the tungsten alloy fragments. These fragments consisted of 91.1% tungsten, 6% nickel, and 2.9% cobalt, similar to the tungsten alloy used in the U.S. tungsten-based kinetic energy penetrators. Tantalum and nickel pellets were purchased from Alfa Aesar (Ward Hill, MA). Tantalum was chosen as the implantation control metal because it is biologically inert (Johansson et al., 1990) with a mass similar to both DU and

tungsten, and it is used frequently in human prostheses (Strecker et al., 1993; Hockley et al., 1990). All pellets were cylinders 1 mm diameter x 2 mm long.

Surgery: Before implantation surgery, DU, tungsten, and tantalum pellets were cleaned and chemically sterilized using the following method. Pellets were washed in an industrial detergent, rinsed in 100% ethanol, soaked in 50% nitric acid for 3 min, rinsed with sterile distilled water, then acetone, and finally air-dried. Anesthesia was induced by continuous administration of isoflurane using an open circuit system with a scavenger/recapture system. Surgical sites were clipped and cleansed with betadine. Pellets were implanted in each gastrocnemius muscle spaced approximately 1.5 mm apart on the lateral side of each leg. Implantation was accomplished by placing the pellet in a 16-gauge needle, putting a specially designed plunger inside that needle, pushing the needle into the rat muscle, then depressing the plunger. This forces the pellet out of the needle and into the rat muscle. Surgical sites were then sutured and sealed with tissue adhesive. This pellet cleaning procedure and surgical technique have been used at AFRRI to implant over 10,000 pellets, without any complications. No rat has shown any signs of infection from the surgery, or any discomfort post-operatively.

Animal observations and assessment of health status: Rats were closely monitored following surgery until they are ambulatory. An analgesic (Buprenorphine) was administered as needed. The surgery sites were examined for signs of inflammation, infection, and local metal-induced toxicity daily for two weeks following surgery and weekly thereafter for the duration of the study.

Task 1 - *Determine whether embedded fragments of DU or WA cause cancer in rodents.*

Specific Methods

Health assessment: Rats were weighed weekly throughout the study and observed once a day for morbidity and mortality. Rats were clinically examined once a week to assess health status.

Clinical pathology: Blood and serum samples were obtained at the time of euthanasia and a hematological and serum chemistry assessment conducted.

Gross pathology: A complete gross pathology was performed on all animals in the study noting any abnormalities. Organ/body weight ratios were calculated to assess overt toxicity of pellet implantation.

. Histopathology: Various tissues were taken for histopathologic examination. Tissues were perfused, embedded, mounted, and stained with hematoxylin and eosin stain (H & E) (Luna, 1968). All tissue changes observed in DU- or WA-implanted rats were contrasted and compared to the identical tissue from control rats.

Task 1 Results

General health assessment: Change in body weight is considered one of the sensitive, early indicators of change in overall health. The animals in this study were weighed weekly. Graphs showing body weight gain over time for all of the experimental groups are shown in Figures 1-6 in the Appendices. Error bars have been omitted for the sake of clarity. In the week immediately after pellet implantation, there is no or only a very slight gain in body weight as the animals recover from surgery. After this recovery period, animals in all groups gain weight at a consistent rate for the next month. After that time, rodents in the high-dose DU group gain weight at a slower rate than the other experimental groups. This decreased rate of body weight gain continues throughout the lifespan of the rat. A similar effect for DU was reported previously for Sprague-Dawley rats (Pellmar et al., 1999). The spikes seen in the graphs of the WA groups, near the end of their lifespan, can be attributed to the low number of animals (one animal in each case) surviving to that point.

No tumors, at the pellet implantation sites, were observed in the tantalum, low DU, or high DU groups at the 1-, 3-, 6-, 12-, 18-, or 24-month time points. Results of gross necropsies are given below. The 18- and 24-month animals, in all surviving groups, exhibited health problems associated with old age (e.g., testicular cancer, benign abdominal growths, etc.). These health problems were not associated with a particular treatment group. They were found across all experimental groups. As a result of these age-related health problems, many of the 18- and 24-month animals were euthanized before reaching their experimental endpoint; however, no abnormalities were observed at the tantalum or DU implantation sites. For the 18-month group, approximately 60 – 70% of the rodents reached the experimental endpoint. Only one rodent (a high-dose DU rat) reached the 24-month experimental endpoint. A table showing survival data for all experimental groups is found in the Appendices (Table 1).

While all the rodents in the non-surgical, tantalum, and DU groups either reached their experimental endpoint or were euthanized due to age-related maladies, the same cannot be said for the WA and nickel groups. By 14-18 weeks after implantation, many of the animals began to develop palpable tumors at the pellet implantation sites. All animals in the low WA, high WA, and nickel groups eventually developed tumors and were euthanized. Criteria for euthanasia are based upon previously published standards (Tomasovic et al., 1988). The high WA group survived the shortest time, with the nickel and low WA groups only slightly longer.

Clinical pathology: Hematological and serum clinical chemistry data for all experimental groups are shown in Tables 2-13 and 14-19, respectively. Several results are noteworthy. Rats implanted with 20 WA pellets exhibited significant increases in white blood cell counts, red blood cell counts, hemoglobin, and hematocrit levels compared to control rats, while rats implanted with 20 Ni pellets had significant decreases in red blood cell counts, hemoglobin, and hematocrit levels. Hematological parameters from low-dose WA rats were not statistically different from controls. The hematological changes observed in the high-dose WA rats are suggestive of polycythemia. Cobalt has been used experimentally to induce polycythemia in rats (Rakusan et al., 2001; Endoh et al., 2000) although the concentration required is far greater than found in the WA pellets. The speed at which these hematological changes occurred in the high-dose WA rats was also surprising. Statistically significant increases in red blood counts, hemoglobin, and hematocrit levels were observed in high-dose WA animals as early as one month after pellet implantation and persisted throughout the experimental period. In addition, there were statistically significant increases in the numbers of neutrophils, lymphocytes, monocytes, and eosinophils present in high-dose WA animals. Low-dose WA animals had elevated neutrophil, lymphocyte, and monocyte numbers, but only the neutrophil numbers were statistically different from the controls. The Ni-implanted animals had significantly lower lymphocyte counts than the controls. All other parameters were statistically identical to the controls. These results suggest a dose-dependent perturbation in many hematology parameters as a result of an increasing WA pellet number.

Gross pathology: Tumors observed during gross necropsy are categorized in Table 20. Gross necropsies showed tumors surrounding the WA or nickel pellets. In many cases the leg tumors would undergo rapid aggressive growth, more than doubling their size in a matter of

days. The tumor appeared to displace and replace the skeletal muscle surrounding the pellet. Metastases were invariably found in the lungs of both the low and high WA groups with additional growths occasionally found in the abdominal cavity. DU and tantalum animals did not exhibit pellet-associated tumors, although many of the rats in the 18- and 24-month groups had abdominal masses and testicular tumors, but these were not specific to a particular treatment. High-dose DU rats in the 24 month group did exhibit a higher number of renal tumors when compared to control. Gross necropsy photomicrographs can be found in our *Environmental Health Perspectives* publication (Kalinich et al., 2005) located in the Appendices.

Metal effects on specific organ systems can often be assessed by measuring organ/body weight ratios. Organ weights and organ/body weight ratios for all experimental groups are found in Tables 21-26. The organs assessed were spleen, thymus, liver, kidney, and testes. Organ weights are provided in the tables, although organ/body weight ratios give a more realistic assessment of organ toxicity. Low-dose DU animals showed an increase kidney/body weight ratio, compared to control, at 1- and 3-months post-implantation. However, by 6 months the ratio had returned to normal and remained there for the duration of the experiment. Surprisingly, the high-dose DU groups did not show these kidney changes, but did show decreases in liver/body weight ratios starting at 3 months post-implantation. These changes persisted throughout the duration of the experiment. The most remarkable changes were seen with the high-dose WA group. Starting at 1 month post-implantation, these animals had extremely elevated spleen/body weight ratios until they were euthanized at approximately 20-24 weeks. In addition, beginning at 3 months post-implantation, kidney/body weight ratios were also elevated.

Histopathology: Histopthological examination of the WA-induced tumors revealed the presence of pleomorphic neoplastic cells with no bone or smooth muscle involvement (Fig. 7). Immunohistochemical analysis was positive for desmin suggesting that the tumor was a rhabdomyosarcoma (Fig 8). There was little or no capsule formation around the WA pellet or associated tumors (Fig. 9). The tumor infiltrated into the skeletal muscle, separating and isolating individual myofibers. Eventually degeneration of the myofibers, with internalization of nuclei, was observed (Figs. 10 and 11). We found that cell pattern and morphology was variable, even within the same tumor (Fig. 12). Cell patterns ranged from

distinct spindle cell in streams with strap-like cells to pleomorphic spindle cell pattern, to a round cell, spider cell and giant cell patterns, more similar to embryonal rhabdomyosarcomas (Figs. 13-17). Tumors formed only around WA and Ni pellets, a condition dramatically illustrated in Fig. 18.

As noted above the high-dose WA rats exhibited characteristics of polycythemia (elevated red blood cell counts, splenomegaly). Shown in Fig. 19 is a cross section of the spleen from one of these rats. There is a noticeable increase in nucleated red blood cells and a mild decrease in the myeloid:erythroid ratio in the red pulp indicative of eyrthroid hyperplasia.

Many of the 18- and 24-month non-surgical, tantalum, and DU (both low and high dose) rats developed interstitial cell tumors of the testes (Fig. 20), as well as abdominal growths identified as chronic nodular granulomatous steatites (Fig. 21). Several DU rats also had adrenal (adrenal medullary, Fig. 22) and renal tumors (tubule carcinoma, mesenchymal tumor, Figs. 23 and 24). These kidney-associated neoplasias were only observed in the 24 month high-dose DU rats, where 5 of 16 rats exhibited abnormal kidney histology.

Task 2 - *Measure tissue levels of DU or WA after chronic in vivo exposure.*

Specific Methods

Sample preparation: At the time of euthanasia a variety of tissues were isolated, blotted dry on gauze, weighed, and placed in 20 ml acid-washed glass scintillation vials along with recovery standard. In addition, serum and urine samples were also obtained at the time of euthanasia and analyzed for metal content. Tissue samples were initially dried in a muffle furnace (Fisher Isotemp Muffle Furnace, Fisher Scientific, Pittsburgh, PA) at 100°C for 24 h. Samples were then dried at 300°C for another 24h, then at 600°C for an additional 48h. Samples were then wet-ashed with 10 ml of 70% nitric acid (Optima Ultrapure Grade, Fisher Scientific) and 200 µl of 30% hydrogen peroxide (Semiconductor grade, Sigma Chemical Co., St. Louis, MO) by heating to just below boiling (to avoid sample splashing) until completely evaporated. After wet-ashing, samples were dry-ashed at 600°C for 8-12 h and then wet-ashed again with nitric acid and hydrogen peroxide. After the second wet-ashing, the residue was dissolved in 2% nitric acid and analyzed. The serum and urine samples did not require the initial dry-ashing step. They were directly wet-ashed with 5 ml of nitric acid and 200 µl of 30% hydrogen peroxide.

The metal content of the various tissue, serum, and urine samples was determined using an inductively coupled-plasma mass spectrometer (PQ ExCell ICPMS System, ThermoElemental, Franklin, MA) equipped with a Cetac ASX500 Autosampler. High-pressure liquid argon, 99.997%, is used for the plasma gas. Instrument operating parameters are given in Table 27. The instrument is calibrated with external standards of 500, 50, 5 and 1 ng/L of the appropriate metal standard in 2% HNO_3. The sample probe is washed with a constant flow of 2% nitric acid between measurements. Quantitative analysis is obtained by reference to the slope of the calibration curve (counts per second / ng per liter) as well as an internal standard. Metal concentration data are normalized, in the case of tissue sample, to gram of tissue analyzed. Serum data are normalized to the volume of serum analyzed and urine data are normalized to creatinine levels.

Task 2 Results

Levels of cobalt, nickel, tungsten, tantalum, and uranium were determined in a variety of tissues (urine, kidney, serum, liver, spleen, muscle) using inductively coupled plasma mass spectrometry (ICP-MS). These data are presented in the Figs. 25-66. Most surprising was the rate at which all three alloy metals (Co, Ni, W) appeared in the urine, particularly tungsten which has been assumed to be relatively insoluble. Maximum urine levels of all three metals were found in the 1 month group, with slightly lower levels found in the 3- and 6-month samples. This indicates that, as with uranium, urine metal levels may prove to be an excellent indicator of exposure to WA. As might be expected, kidney levels of all metals were elevated, showing a dose- and time-dependence. Serum levels of cobalt, nickel, and tungsten mirrored the pattern seen for urine from WA-implanted animals. Serum metal analysis may also prove to be a good indicator of exposure. DU was only present at very low levels in the serum, supporting results obtained by others. Both liver and spleen contained all three of the alloy metals, with significantly high values for tungsten at the 6 month time point. DU was present at very low levels in both spleen and liver. Muscle (gastrocnemius, sampled close to the implantation sites) metal levels were also very low for all metals indicating very little diffusion of the metals through the muscle.

Task 3 - *Assess the genotoxicity and mutagenicity after chronic in vivo exposure to DU or WA.*

Specific Methods

Sample collection: At 6, 12, 18, and 24 months after metal implantation, blood and urine were collected from rats in all experimental groups

Genotoxicity - peripheral blood lymphocyte model system: Peripheral blood lymphocytes (PBL) were isolated from whole blood for short-term culture on a density gradient (Histopaque, Sigma Chemical Co.). Isolated PBL were cultured according to a standard protocol (Kligerman et al., 1981). PBL cultures containing between 1 to 1.5 x 10^6 cells were resuspended in a complete growth medium (Karyomax, Life Technologies, Rockville, MD), stimulated to divide by the addition of phytohemagglutinin (Murex Diagnostics, Ltd, Hartford, UK), and incubated at 37 °C for varying durations for different cytogenetic assays.

Genotoxicity - preparation of metaphase spreads: After 24 h of culture initiation, 5-bromodeoxyuridine (Sigma Chemical Co.) was added to differentiate between first- and second-division metaphase spreads. First- and second-division metaphase spreads were harvested following an additional incubation at 37 °C and a 4-h treatment with colcemid to arrest cell cycle progression in metaphases. Following a hypotonic treatment in 1% sodium citrate solution, the cells were fixed in 1:3 acetic acid:methanol fixative and metaphase spreads prepared on acid-cleaned microscope slides by the standard method. The first- and second-division metaphase spreads were used for chromosome aberration and SCE analysis respectively, as described below.

Genotoxicity - chromosome aberration analysis: The frequency of various types of chromosome aberrations including dicentrics were estimated in first-division metaphase spreads following differential staining using the standard fluorescence plus Giemsa staining protocol (Perry and Wolff, 1974). Briefly, the slides were treated with Hoechst 33258 (Sigma Chemical Co.) for approximately 30 min, rinsed, immersed in Sorenson's buffer (pH 6.8) for 15 – 20 min at 56 °C, and exposed to a black light (360 nm). Slides were rinsed in a buffer and counterstained with 4% Giemsa in Sorenson's buffer (pH 6.8) to differentiate, using light microscopy, first- and second-division metaphase spreads.

Genotoxicity - SCE analysis: The frequency of SCE was estimated in second-division metaphase spreads following the differential staining procedure as described above.

Genotoxicity - micronucleus assay: For the analysis of micronuclei in PBL, the cytokinesis-blocked micronucleus technique of Fenech and Morley (1985) was used. Briefly, 44 h after the start of the lymphocyte culture, cytochalasin B (Sigma Chemical Co.) was added to a final concentration of 3 μg/ml. After incubating at 37 °C to obtain binucleated cells, cytocentrifuge preparations of the PBL were made on clean slides. The cells were allowed to dry and then fixed in absolute methanol, before staining with May-Grunwald-Giemsa and analyzing by light microscopy. Scoring of micronuclei was limited to binucleate cytokinesis-blocked cells.

Serum and urine mutagenicity: Collected urine was passed over chromatographic columns containing Amberlite XAD-4 resin (Rohm and Haas, Philadelphia, PA). The effluents were collected and passed through a column packed with Amberlite XAD-8 resin. Resins were purified by Soxhlet-extraction before use. Use of these columns allows for the collection of a hydrophobic urine fraction (XAD-4) and a hydrophilic fraction (XAD-8). The column was successively washed with 10 ml of distilled water and eluted with acetone. Dry extracts were dissolved in DMSO (320 μl). This process resulted in a 100X concentrate.

For the serum mutagenicity studies, blood was obtained as described above and serum separated by centrifugation (5 min at 3,000 x g) prior to vacuum-dialysis through a dialysis-tube with a cutoff value of 30,000 Daltons.

To assess urine and serum mutagenicity, the *Salmonella* bacterial reversion assay, developed by Maron and Ames (1983), was used. The Ames II™ mutagenicity assay by Xenometrix (Boulder, CO) was used for these assessments. The two bacterial strains used were the Ames II™ mixed strains and the frameshift tester strain TA98. AmesII™ mixed strains contain equal numbers of the cells of each of the six strains (TA7001-7006). Individually, these strains revert by only one specific base-pair substitution out of six possible changes. Therefore, when mixed, all six base substitution mutations can be represented in one culture (Gee et al., 1994). Aliquots of 10 μl of urine extracts or 1000 μl of dialyzed sera were incorporated into three plates inoculated with either Salmonella strains TA98 or the Ames II™ TA7001-7006 strains without metabolic activation. The dose of the urine tested was 1 ml equivalent (ml-eq) per plate. Samples were assessed with positive and negative controls (107) and several revertant

colonies were randomly selected for verification. The results are expressed as revertants/µl of sample.

Task 3 Results

Examination of chromosomal aberrations in peripheral blood lymphocytes suggests that embedded WA or DU is not genotoxic (a typical chromosome spread is shown in Fig. 67). There was also no indication that serum from any of the experimental groups at the four timepoints measured (6, 12, 18, and 24 months) was mutagenic when assayed by the Ames bacterial reversion assay (Table 28). However, urine from the high-DU and high-WA groups was significantly more mutagenic than urine from non-surgical or tantalum implanted rats. This result was not unexpected as one of the major routes of excretion of these metals from the body is in the urine. No urine was collected from the 18- and 24-month animals because, due to the declining health of the rodents, we opted against stressing the animals further by housing them in the metabolic cages required for urine collection.

Task 4 - *Determine the effect of embedded DU and WA on the organs of the immune system.*

Specific Methods

Immune organ weights and cellularity: One, 3, 6, and 12 months post-implantation, rats were weighed, euthanized, and the spleen and thymus sterilely removed and placed in RPMI-1640 medium with 10% fetal bovine serum (RPMI Complete) (Gibco Life Technologies, Gaithersburg, MD). Excess medium was removed from each thymus and spleen by blotting onto sterile dry gauze before weighing the organ. Single-cell suspensions were prepared from thymus and spleen by placing the tissue in a "polybag" with 7 ml of RPMI Complete and crushing it in a Stomacher 80 Lab-Blender (Tekmar Corporation, Cincinnati, OH) for 10 min. The homogenate was then passed through sterile nylon mesh (Spectrum Medical Industries, Houston, TX) to remove tissue debris, the polybag rinsed with an additional 8 ml of RPMI Complete, and the suspension centrifuged for 10 min at 300 x g and 4^{o}C. The resulting pellet was washed with RPMI Complete before resuspension and determination of cell number using a Coulter Counter (Model ZM, Hialeah, FL). For the determination of bone marrow cellularity, femurs were

removed, cleaned of all muscle and tissue with sterile gauze, both ends removed, and the marrow flushed with 5 ml of RPMI Complete using an 18-gauge needle. The cell suspension was transferred to centrifuge tube, titurated 8-10 times through a 21-gauge needle, then 3-5 times through a 25-gauge needle before being passed through sterile nylon mesh in order to remove bone fragments. The filtered suspension was centrifuged as above and cell number determined as for thymus and spleen.

Blood was collected from the abdominal aorta and a total hematology analysis conducted. Parameters included white blood cell counts, lymphocytes (% and absolute), monocytes (% and absolute), granulocytes (% and absolute), red blood cell counts, hemoglobin, hematocrit, and platelet counts.

Phenotypic analysis by flow cytometry: Phenotypic analysis of both spleen cell suspensions and peripheral blood was accomplished by flow cytometry. Single-cell splenocyte suspensions were prepared as described above. The cells were washed once with "wash buffer" (Dulbecco's phosphate buffered saline with 0.1% sodium azide), the cell pellet resuspended in 1 ml of wash buffer before adding 14 ml of "lysis buffer" (15 mM ammonium chloride, 1 mM potassium bicarbonate, 0.008 mM EDTA), and the suspension incubated for 5 min at room temperature in order to lyse any contaminating erythrocytes. The suspension was centrifuged (300 x g for 5 min at room temperature), washed once with wash buffer, and the cell pellet resuspended in wash buffer supplemented with 2% heat-inactivated fetal bovine serum. After cell number and viability determination using the trypan blue dye exclusion technique, the cells were resuspended to a final concentration of 5×10^6 cells/ml.

Peripheral blood was collected from the abdominal aorta into blood collection tubes containing EDTA (Vacutainer, Becton-Dickinson, Franklin Lakes, NJ). In order to lyse erythrocytes, 1 ml of peripheral blood was mixed with 14 ml of lysis buffer and left at room temperature for 5 min. The mixture was then centrifuged (300 x g for 5 min at room temperature), the cell pellet washed with wash buffer, and finally resuspended in wash buffer supplemented with 2% heat-inactivated fetal bovine serum. After determination of cell number and viability using the trypan blue dye exclusion technique, the cells were resuspended to a final concentration of 5×10^6 cells/ml.

For both splenocytes and peripheral blood lymphocytes, 50 μl of cell suspension was incubated with 10 μl of the appropriate fluorescently labeled cell surface antibody in the dark at 4^{o}C for 30 min. After this time, 3 ml of wash buffer was added, and the cells centrifuged (300 x g for 5 min at 4^{o}C). The cell pellet was resuspended in 1 ml of "wash buffer" containing 2% fetal bovine serum before analysis by flow cytometry. The following antibodies (all from BD Biosciences, San Diego, CA) were used. Phycoerythrin-labeled OX-19 monoclonal antibody was used to detect the CD5 marker on T cells. Fluorescein isothiocyanate (FITC)-labeled OX-38 monoclonal antibody was used to detect the CD4 antigen on T helper/amplifier cells and macrophages. T cytotoxic/suppressor cells and natural killer (NK) cells, which express the CD8 marker, were detected using FITC-labeled OX-8 monoclonal antibody. B cells were detected with FITC-labeled OX-33, a monoclonal antibody specific for CD45 and found only on B cells. Non-viable cells were detected by propidium iodide (1 μg/ml, Sigma Chemical Co., St. Louis, MO) staining, gated, and excluded from analysis. The labeled cells were analyzed with a FACScan Flow Cytometry System (Becton-Dickinson Immunocytometry Systems, Mountain View, CA) using the associated Cell Quest Color Analysis operating system.

Task 4 Results

Assessments for this task included immune organ/body weight ratios (Tables 21-24), immune organ cellularities (Tables 29-32), hematological assessments (Tables 2-9), and flow cytometric analysis of peripheral blood, splenocytes, and thymocytes (Tables 33-36). Fisher 344 rats implanted with 4 (low dose) or 20 (high dose) pellets of DU or WA were assessed at 1, 3, 6, and 12 months post-implantation. (Note: Because of aggressive tumor formation in the WA-implanted rats, no animals survived to 12 months).

Immune organ/body weight ratio: Organ/body weight ratios are often sensitive indicators of toxicity. Fisher 344 rats implanted with 4 (low dose) or 20 (high dose) pellets of DU showed no significant differences in spleen/body weight or thymus/body weight ratios when compared to control rats. Rats implanted with 20 pellets (high dose) of WA showed significantly higher spleen/body weight ratios than control rats, indicating splenomegaly. The spleen/body weight ratios of rats implanted with 4 pellets (low dose) of WA, although elevated, were not statistically different then control rats. The thymus/body weight ratios of WA-implanted rats were not

significantly different than control rats, with the exception of the high-dose WA group at 5-6 months post-implantation.

Immune organ cellularity: The number of cells per gram of spleen and thymus, as well as the number of bone marrow cells per femur, were determined. For WA- implanted rats, spleen cellularity at 1 month post-implantation was significantly lower than in control rats in both the low- and high-dose groups; however, these numbers returned to normal by 3 months post-implantation. Thymus cellularity in WA-implanted animals was no different than controls, except for the 6 month low-dose group, and there were no differences in bone marrow cellularities in any of the WA groups when compared to control. Low-dose DU implanted rats showed lower splenocyte and thymocyte counts at 6 months post-implantation, although the high-dose DU implanted rats did not show these decreases. However, bone marrow counts in both low- and high-dose DU rats were significantly lower starting at 3 months post-implantation and stayed lowered throughout the 12 month experimental time course.

Hematology: Complete hematologic assessments were conducted on all animals. DU-implanted rats showed very few significant and lasting changes. Low-dose DU rats had higher white blood cells and lymphocyte counts (compared to control) at 1 month post-implantation. However, these numbers were then lowered than control at the 3 month timepoint. Neutrophil counts were also lower in low-dose DU rats at 3 months. By 6 months post-implantation, these values had returned to normal. In high-dose DU rats, platelet counts were elevated, compared to control, at both 1 and 3 months post-implantation. They returned to normal at 6 month; however, both white and red blood cell counts, as well as hematocrit levels, were depressed. These values returned to normal at 12 months post-implantation. In both the low- and high-dose DU rats, platelet values were lower than control at 12 months post-implantation.

A variety of hematological changes were observed in WA-implanted rats. For low-dose WA rats, the hematological changes, including significant increases in red blood cells, white blood cells, hemoglobin, hematocrit, neutrophils, lymphocytes, and monocytes, peaked at 3 months post-implantation and returned to normal by 5-6 months. High-dose WA rats demonstrated the same changes observed in low-dose WA rats, but they occurred much more rapidly (as early as 1 month post-implantation) and persisted throughout the life of the animal. The splenomegaly and hematological changes observed in these rats are suggestive of

polycythemia. These results suggest a dose-dependent perturbation in many hematology parameters as a result of an increasing WA pellet number.

Flow cytometry: Flow cytometric analysis of peripheral blood lymphocytes, splenocytes, and thymocytes was conducted in order to investigate any changes, as a result of prolonged exposure to embedded DU or WA, in the subpopulations of cells that comprise these immune system organs. Using the methods of Flaherty et al. (1997) and Capri et al. (2000), the CD4 and CD8 subpopulations in isolated thymocytes; the cytotoxic T-cell, T-helper cell, and the naïve and activated subpopulation of CD4 and CD8 cells in peripheral blood; and the T cell, B cell, cytotoxic T cell, T helper cell, NK cell, and putative monocyte levels in isolated splenocytes were determined.

For DU-implanted rats, the number of splenic NK cells were decreased 1 month post-implantation, but had returned to normal by 3 months post-implantation. All assessments at every other time point were not statistically different than control, with the exception of the 6-month splenic NK cells for the low-dose DU group which were significantly higher than control. However, at 12 months post-implantation there was no significant difference and the high-dose DU group showed no such changes.

For WA-implanted rats, there were numerous and consistent changes in immune cell subpopulations in both peripheral blood and spleen. High-dose WA-implanted rats showed significant decreases in peripheral blood cytotoxic T cells, T helper cells, and naïve CD4 and CD8 cells starting as early as 1 month post-implantation. Both the low- and high-dose WA rats demonstrated lower splenic B cell levels, as well as lower NK cell levels (as did both DU groups) at 1 month post-implantation. The peripheral blood subpopulation changes persisted in the high-dose WA rats at 3 months post-implantation. The splenic B cell levels returned to normal in both WA groups, but splenic NK cell levels remained depressed. By 6 months post-implantation, all WA rats had developed tumors at the pellet implantation site and were reaching the criteria for euthanasia. Not surprisingly, all peripheral blood flow cytometric parameters were significantly lower in both the low- and high-WA animals as compared to control. Somewhat surprisingly, values for the various immune system cell subpopulations in the spleen were not statistically different from control, except for splenic NK cell levels which had risen significantly higher. As noted earlier, no WA animal survived to the 12 month time point.

Task 5 – *Evaluate the effect chronic in vivo exposure to DU and WA has on immune function, including cell-mediated, humoral, and innate immunity.*

Specific Methods

The effect of embedded DU and tungsten alloy on cell-mediated immune response was assessed by the cytotoxic T-lymphocyte (CTL) assay. Splenocytic CTL induction was determined using the murine mastocytoma cell line P815 as the target cell. Tissues were harvested sterilely and single-cell suspensions prepared as described in Task 4. The splenocytes were washed in Dulbecco's phosphate buffered saline (D-PBS) containing 1% fetal bovine serum (FBS), then resuspended in 2 ml of "lysis buffer" (144 mM ammonium chloride; 17 mM Tris, pH 7.65) and incubated at room temperature for 5 min in order to lyse any contaminating erythrocytes. The cells were then pelleted (300 x g for 5 min at room temperature), washed twice with D-PBS/1% FBS, and resuspended in culture medium [RPMI-1640 supplemented with 10% FBS, 2 mM glutamine, penicillin/streptomycin, 5 μM 2-mercaptoethanol, and 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)]. After determining cell number and viability using the trypan blue dye exclusion technique, cell density was adjusted to 6×10^7 cells/ml and aliquoted to tissue culture flasks at a concentration of 3×10^7 cells/flask along with 19 ml of culture medium. The P815 murine mastocytoma cell line (TIB-64, American Type Culture Collection, Manassas, VA) was maintained in mid-log phase growth in Dulbecco's modified Eagle's medium supplemented with 4 mM glutamine, 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate, 4.5 g/l glucose, penicillin/streptomycin and 10% fetal bovine serum. Prior to use, the cells were pelleted by centrifugation (300 x g for 5 min at 4^oC), resuspended in 5 ml of culture medium, and cell number and viability determined using the trypan blue dye exclusion technique. Mitomycin C was added to a final concentration of 25 $\mu g/10^7$ cells and the cells incubated in a 37^oC shaking water bath (45 rpm) for 30 min in the dark. After this, the cells were washed three times with D-PBS containing 5% FBS, resuspended in D-PBS/5% FBS, and left at room temperature for 20 min. The cells were then pelleted by centrifugation, washed again with D-PBS/5% FBS, resuspended in culture medium [RPMI-1640 supplemented with 10% FBS, 2 mM glutamine, penicillin/streptomycin, 5 μM 2-mercaptoethanol, and 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)], cell number and viability

determined by the trypan blue dye exclusion technique, and the cell density adjusted to 1.2 x 10^6 cells/ml. The P815 cells (6 x 10^5 cells/flask) were then added to each flask containing the splenocytes and all flasks were incubated at 37°C in a 5% CO_2/95% air incubator. After 5 days, the cells were collected by centrifugation (300 x g for 5 min at room temperature), resuspended in culture medium, cell number and viability determined by trypan blue dye exclusion, and the cell density adjusted to 5 x 10^6 cells/ml. Serial dilutions (5 x 10^6 - 5 x 10^3 cells/ml) were made in culture medium and 100 μl of each dilution added to quadruplicate wells of a 96-well plate. P815 cells from another log-phase culture were pelleted by centrifugation (300 x g for 5 min at room temperature), resuspended in culture medium, cell number and viability determined by trypan blue dye exclusion, the cell density adjusted to 2 x 10^5 cells/ml, 100 μl of the cell suspension added to each well of the 96-well plate, and the plate centrifuged (250 x g for 4 min at room temperature). The Promega CytoTox 96® Non-Radioactive Cytotoxicity Assay, which colorimetrically measures lactate dehydrogenase (LDH) release, was used instead of ^{51}Cr to determine cytotoxicity. The results obtained with this system have been shown to be identical to results obtained with ^{51}Cr release assays (Korzeniewski and Callewaert, 1983; Decker and Lohmann-Matthes, 1988). Briefly, the plate was incubated at 37°C in a 5% CO_2/95% air incubator for 4h, centrifuged (250 x g for 4 min at room temperature), and 50 μl aliquots from each well transferred to a new 96 well flat-bottom plate. “Assay Buffer” (12 ml, supplied in the kit) was mixed with one bottle of “Substrate Mix” (also supplied in the kit). To each well of the 96-well plate, 50 μl of this “Substrate Mixture” was added and the plate incubated in the dark for 30 min at room temperature. After the incubation period, 50 μl of “Stop Solution” (provided in the kit) was added to each well and the absorbance read at 490 nm. By including the proper controls, the percent cytotoxicity was calculated:

$$\% \text{ Cytotoxicity} = \frac{\text{experimental} - \text{effector spontaneous} - \text{target spontaneous}}{\text{target maximum} - \text{target spontaneous}} \times 100$$

where “experimental” is the absorbance of the well containing both P815-stimulated splenocytes and target P815 cells, “effector spontaneous” is the absorbance obtained from wells containing

P815-stimulated splenocytes alone, “target spontaneous” is the absorbance obtained from wells containing P815 cells alone, and “target maximum” is the absorbance obtained from wells containing P815 cells that have been lysed with Triton X-100 (represents the total amount of LDH in the target cells).

Humoral immunity was assessed using the antibody plaque-forming cell assay. Four days prior to sacrifice, rats were injected with sheep red blood cells (2×10^8 cells in 1 ml of Earle’s balanced salt solution). Prior to euthanization, a 0.5% agar/0.05% DEAE-dextran solution was prepared, 0.5 ml aliquots of this mixture placed in prewarmed borosilicate glass culture tubes (12 x 75 mm), and the tubes kept in a 48°C water bath until needed. Spleens were harvested from control and implanted animals and sterile single-cell suspensions prepared as described in Task 4. The cell suspensions were washed, resuspended in 6 ml of Earle’s balanced salt solution with HEPES buffer, and diluted 1:50 and 1:150. For plaquing, sheep red blood cells were washed three times with Earle’s balanced salt solution without HEPES, resuspended in 50% of the original volume with Earle’s balanced salt solution with HEPES, and 25 μl of this added to the culture tubes. Then, 100 μl of the spleen cell suspension was added and the tubes removed from the water bath, combined with 25 μl of a 1:4 dilution of ice-cold stock guinea pig complement, the tubes vortexed, the mixture poured into 100-mm Petri dishes and covered with coverslips, and the agar allowed to solidify. The plates were then incubated at 37°C for 3 h. During this time, cell counts on the original splenocyte suspensions were obtained. After the 3-h incubation, the number of plaques was counted and results reported as number of plaque-forming cells (PFC) per spleen and number of PFCs per 10^6 cells.

The effect of embedded DU and WA on the innate immune response was determined by assessing natural killer (NK) cell activity. Briefly, single-cell suspensions were prepared from spleens of control and implanted animals as detailed in Task 4. After washing the suspensions with cold complete culture medium (RPMI supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, 25 mM HEPES, 50 μM 2-mercaptoethanol, and 10% FBS), the pellet was resuspended in 5 ml of cold complete culture medium, a 50 μl aliquot removed and mixed with 400 μl of sterile “ACK” buffer (150 mM ammonium chloride; 1 mM potassium bicarbonate; 0.1 mM disodium EDTA; pH 7.2) to lyse erythrocytes, and 50 μl of trypan blue added in order to determine cell number and viability. Cell density was adjusted to 2×10^6

cells/ml with cold complete culture medium. The target cell for this assay is the mouse lymphoma line, YAC-1 (TIB-160, American Type Culture Collection). The cultures were maintained in RPMI-1640 medium supplemented with 2 mM glutamine and 10 % FBS and incubated at 37°C in an atmosphere of 5% CO_2/95% air. Only cells in log-phase growth were used for the NK assay. The YAC-1 cells were pelleted by centrifugation (200 x g for 10 min at room temperature) and resuspended in fresh complete culture medium. Cell number and viability were assessed by trypan blue dye exclusion and the final density of YAC-1 cells adjusted to 5 x 10^4 cells/ml with complete culture medium. To assay for NK function, serial dilutions of the splenocyte suspension were done to yield splenocyte/target cell ratios of 200:1, 100:1, 50:1, and 25:1, with the volume of splenocyte suspension kept constant at 100 µl. The cell suspensions were added to a 96-well plate in triplicate. The YAC-1 cells were then added (100 µl/well of 5 x 10^4 cells/ml) and the plate centrifuged (250 x g for 4 min at room temperature). As described above, the Promega CytoTox 96® Non-Radioactive Cytotoxicity Assay, which colorimetrically measures lactate dehydrogenase (LDH) release, was used instead of ^{51}Cr to determine cytotoxicity.

Task 5 Results

Three tests of immune function were conducted: the cytotoxic T-lymphocyte assay, the Natural Killer (NK) cell assay, and the antibody plaque-forming cell assay.

Cytotoxic T-Lymphocyte Assay : Cytotoxic T-lymphocytes (CTL) are an important component of the adaptive immune response, especially with respect to intracellular pathogens. In low-dose DU implanted rats, CTL activity is no different than control at 1 month post-implantation, is elevated at 3 months, slightly depressed at 6 months, and returns to normal at 12 months post-implantation. In high-dose DU rats, CTL activity is slightly depressed at 6 months post-implantation, but is no different than control at all other times. In low-dose WA rats, CTL activity is no different than control at 1 and 3 months post-implantation, but is lower at 6 months (the final time point assayed). Similar results are seen for high-dose WA rats except they also exhibit depressed CTL activity at 3 months post-implantation. Data are shown in Table 37.

Natural Killer Cell Assay : Natural killer (NK) cells are lymphocytes involved in the early immune response to both viral pathogens and malignant transformation. In rodents, NK

activity is highest when the rats are young and rapidly decreases to non-detectable levels later in life. We also found that in this study. There was no detectable NK activity at the 6- and 12-month timepoints. In low-dose DU rats at 1 month post-implantation, NK activity is below that of control rats. At 3 months, NK activity is still low but it is not statistically different than control. In high-dose DU rats, NK activity is normal at 1 month, but lower than control groups at 3 months post-implantation. In both low- and high-dose WA rats, NK activity is significantly depresses compared to control rats at both the 1 and 3 month timepoints. Data are shown in Table 38.

Antibody Plaque-forming Cell Assay : The antibody plaque-forming cell assay (APC) measures the ability of the animal to elicit an antibody response to a foreign antigen. It is considered the "gold standard" test for immunotoxicity assessments. In DU-implanted rats, APC activity was no different than control except for two points: the 12 month point for low-dose DU rats and the 6 month point for high-dose DU rats were both lower than control groups. For WA rats, the low-dose group was no different than control at 1 month, but then exhibited elevated APC activity at 3 and 6 months post-implantation. (No rats survived to the 12 month timepoint). For high-dose WA rats, APC activity was no different than control at 1 month, but then was significantly elevated at 3 months post-implantation. Data are shown in Table 39.

KEY RESEARCH ACCOMPLISHMENTS

- All animals implanted with WA (both low and high groups) or nickel developed tumors, identified as rhabdomyosarcomas, at the implantation site.
- Tumor development occurred most rapidly in the high WA group.
- Tumors metastasized to the lung in both the low and high WA groups.
- Histopathological examination showed that the tumors exhibited characteristics of both classical or pleomorphic rhabdomyosarcomas as well as embryonal rhabdomyosarcomas.
- No pellet-implantation site tumors were found in the tantalum- or DU-implanted animals.
- High-dose DU rats in the 24 month group exhibited a variety of kidney neoplasias
- Significantly high levels of cobalt, nickel, and tungsten appeared in the urine and serum of WA-implanted rats as early as one month after pellet implantation.

- Rodents in the high WA group exhibited splenomegaly and hematological changes suggesting polycythemia.
- Rodents in the high WA group exhibited altered distribution of lymphocyte subpopulations in both peripheral blood and spleen as early as one month after pellet implantation.

REPORTABLE OUTCOMES

Oral Presentations and Abstracts

Oral presentation (and abstract) by Dr. David McClain at the Force Health Protection Conference (Albuquerque, NM, 11-15 August 2003). Title: Health Effects of Embedded Depleted Uranium and Tungsten Alloy Fragments in Rats.

Oral presentation by Dr. David McClain at the US Army Heavy Metals Office Heavy Alloys Workshop, Stevens Institute of Technology, Hoboken, NJ, 10-11 February 2004. Title: Tumor Induction in Rats by Embedded Tungsten Alloy Fragments.

Oral presentation by Dr. David McClain at the United States/United Kingdom International Exchange Agreement 1443 Workshop (US/UK IEA 1443), US Army Research Laboratory, Aberdeen, MD, 24 June 2004. Title: Tumor Induction in Rats by Embedded Tungsten Alloy Fragments

Oral presentation by Dr. David McClain at the Armed Forces Radiobiology Research Institute Seminar Series, 5 March 2004. Title: Investigation of Tumor Induction in Rats by Embedded Tungsten Alloy Fragments.

Oral presentation by Dr. David McClain at the Armed Forces Radiobiology Research Institute Scientific Program Overview, AFRRI Board of Governors Meeting, 27 July 2004. Title: Investigation of Health Effects of Embedded Tungsten Alloy Fragments in Rats.

Oral presentation by Dr. John Kalinich at the Armed Forces Radiobiology Research Institute Seminar Series, March 19, 2004. Title: Immunotoxic Potential of Militarily Relevant Heavy Metals.

Oral presentation by Dr. John F. Kalinich at the TSCA ITC Meeting, Washington, DC, May 5, 2005. Title: Health Effects of Tungsten Alloys.

Oral presentation (and abstract) by Dr. David McClain at the NATO RTG-099 Meeting, Bethesda, MD, June 21-23, 2005. Title: Status of Health Concerns about Military Use of Depleted Uranium and Surrogate Metals in Armor Penetrating Munitions.

Oral presentation (and abstract) by Dr. David McClain at the 8th Annual Force Health Protection Conference, Louisville, KY, August 7-12, 2005. Title: Embedded Tungsten Alloy Fragment Research and its Implications.

Oral presentation (and abstract) by Dr. John Kalinich at the American Chemical Society 230th National Meeting, Washington, DC, August 28 – September 1, 2005. Title: Embedded Weapons-grade Tungsten Alloy Shrapnel Rapidly Induces Metastatic High-grade Rhabdomyosarcomas in F344 Rats.

Oral presentation by Dr. David McClain at the Military Health Services Tricare Convention, Arlington, VA, 01 February 2006. Title: Carcinogenicity of Embedded Tungsten Alloy in Rats.

Oral presentation (and abstract) by Dr. David McClain and Dr. John Kalinich at the 2006 International Conference on Tungsten, Refreactory and Hardmetals VI, Orlando, FL, 7-8 February 2006. Title: Embedded Weapons-grade Tungsten Alloy Shrapnel Rapidly Induces Metastatic High-grade Rhabdomyosarcomas in F344 Rats,

Oral presentation (and abstract) by Dr. John Kalinich at the U.S. Army Heavy Metals Office's Heavy Metals Forum, Baltimore, MD, 7-9 March 2006. Title: Carcinogenicity of Embedded Tungsten Alloy in Rats – Observations and Outlook.

Oral presentation by Dr. John Kalinich at the Armed Forces Radiobiology Research Institute Seminar Series, March 17, 2006. Title: Health Effects of Embedded Tungsten Alloy Fragments

Oral presentation by Dr. John Kalinich at the Tungsten Hazards Meeting, Picatinny Arsenal, NJ, 21 March 2006. Title: Health effects of embedded tungsten alloy in rats.

Annual Reports

Alexandra C. Miller, John F. Kalinich, and David E. McClain, Carcinogenicity and immunotoxicity of embedded depleted uranium and heavy-tungsten alloy in rodents. Annual Report 17Sept2001-16Sept2002, 17 pg, DTIC Accession Number: ADA409697.

John F. Kalinich, Alexandra C. Miller, and David E. McClain, Carcinogenicity and immunotoxicity of embedded depleted uranium and heavy-tungsten alloy in rodents. Annual Report 17Sept2002-16Sept2003, 98 pg, (limited distribution).

John F. Kalinich, Alexandra C. Miller, and David E. McClain, Carcinogenicity and immunotoxicity of embedded depleted uranium and heavy-tungsten alloy in rodents. Annual Report 17Sept2003-16Sept2004, 51 pg, DTIC Accession Number: ADA428281.

John F. Kalinich, Alexandra C. Miller, and David E. McClain, Carcinogenicity and immunotoxicity of embedded depleted uranium and heavy-tungsten alloy in rodents. Annual Report 17Sept2004-16Sept2005, 34 pg, DTIC Accession Number: ADA442322.

Manuscripts

John F. Kalinich, Christy A. Emond, Thomas K. Dalton, Steven R. Mog, Gary D. Coleman, Jessica E. Kordell, Alexandra C. Miller, and David E. McClain, Embedded weapons-

grade tungsten alloy shrapnel rapidly induces metastatic high-grade rhabdomyosarcomas in F344 rats, Environmental Health Perspectives **113**: 729-734 (2005).

David E. McClain, Alexandra C. Miller, and John F. Kalinich, Status of health concerns about military use of depleted uranium and surrogate metals in armor-penetrating munitions, Proceedings of the Human Factors and Medicine (HFM) Panel Research Task Group (RTG) 099 Meeting, Bethesda, MD June 21-23, 2005.

Two additional manuscripts are in preparation. Copies will be sent to USAMRMC for the files once the manuscripts are accepted for publication.

Personnel Employed Under This Grant:

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CONCLUSIONS

Project Summary

This study investigated the carcinogenic and immunotoxic potential of embedded fragments of depleted uranium (DU) and a heavy-metal tungsten alloy (WA). Male Fisher 344 rats were surgically implanted with pellets of DU, WA, tantalum (inert metal, negative control), or nickel (known carcinogen, positive control). Implanted WA resulted in the rapid formation of tumors, identified as rhabdomyosarcomas, surrounding the pellets. These tumors had, within the same area, histopathological characteristics of both the pleomorphic and embryonal subtypes of rhabdomyosarcomas. Eventually these tumors metastasized to the lung. Tumor incidence in the WA-implanted rats was 100%. Rats implanted with tantalum or DU pellets did not develop tumors at the implantation site. However, several rats (5 of 16) in the 24 month high-dose DU group did exhibit renal neoplasias. No renal tumors were seen in the control groups.

WA-implanted rats (high-dose group) also exhibited splenomegaly and hematological changes suggesting polycythemia as early as 1 month after pellet implantation. The low-dose WA-implanted rats also showed elevated spleen/body weight ratios and increased RBC, hemoglobin, and hematocrit levels, but these changes were not considered statistically different from control. These results suggest that there may be a dose-dependence with respect to the observed WA-induced polycythemia.

Although only approximately 5% of the 35 mg total weight of a WA pellet was lost as a result of being implanted for 6 months, elevated levels of tungsten, nickel, and cobalt rapidly

appeared in the urine and serum of WA-implanted animals, with significant levels seen as early as one month post-implantation. Urine and serum levels may prove to be reliable indicators of tungsten alloy exposure. Elevated levels of all three alloy metals were also found in the kidney. As expected, elevated levels of DU were found in the urine and kidney of both the low- and high-dose groups.

Exposure to embedded DU pellets had only transient effects on immune system parameters with the notable exception of bone marrow cellularity. Cell numbers in bone marrow from the femurs of both low- and high-dose DU groups were significantly lower than controls starting at three months post-implantation. The cell numbers stayed significantly lower through the 12 month time point. On the other hand, WA exposure had significant effects on multiple parameters of immune system integrity. WA implantation resulted in significantly lower levels of a variety of peripheral blood and splenic immune system cells. In addition, tests of cell-mediated and innate immunity were significantly lower in the WA-implanted groups, while the antibody-plaque cell assay, a test of humoral immunity, was significantly higher in WA-implanted rats. These results suggest that embedded WA is immuntoxic, although the observed immuntoxicity is secondary compared to the carcinogenicity.

Significance of Findings to Military Health Issues

Tungsten alloy: The finding from this study that embedded WA pellets resulted in rapid and aggressive tumor development has raised significant concern since heavy-metal tungsten alloys are being used as replacements for DU in armor penetrators. In fact, many countries, some unfriendly to the U.S., already possess tungsten munitions. As a result, future combat could produce large numbers of U.S. personnel with tungsten fragment injuries with military surgeons not having the best information available to deal with those injuries.

It should be pointed out that the tungsten alloy we used consisted of tungsten, nickel, and cobalt. Other tungsten alloys, tungsten/nickel/iron in particular, are also being used to produce armor penetrating munitions. Because of the lack of information on the health effects of embedded fragments composed of these types of mixtures, investigations on potential tumor development patterns for implanted tungsten/nickel/iron, as well as for the individual metals alone and in various combinations are sorely needed. With these data, a determination of the

carcinogenic potential of the individual metals can be made, as well as a determination of any synergistic effects that may occur when the various metals are present together. With these results, a comparison of the various alloys can be made and the exposure risk put into perspective.

Another critical result from this study is the finding that tungsten, nickel, and cobalt levels in the urine and serum of WA-implanted rats are significantly elevated as early as 1 month after pellet implantation (the earliest time measured). This discovery could provide the basis for an early detection protocol for WA exposure, but only if additional research is conducted to investigate how soon after pellet implantation the alloy metals appear in the urine and serum.

Depleted uranium: Embedded DU was shown not to be carcinogenic. However, a finding from this study that could have serious consequences is the significant increase in the number of renal neoplasias in the 24 month high-dose DU group. Although at the present time there have been no renal or clinical abnormalities reported in DU-wounded veterans from the first Gulf War (McDiarmid et al., 2006), subtle changes in renal function have been reported for those individuals with the highest urine uranium concentrations (Squibb and McDiarmid, 2006). More work in this area is clearly needed.

Critical Future Investigations

One important result of this research is that it points out the need for health-effects studies on militarily relevant material prior to deployment to the field. We highly recommend that a screening protocol be implemented to assess potential health effects of new munition material prior to their widespread use. In addition, there are several research areas that we believe require immediate attention:

- Carcinogenic and immunotoxic assessment of embedded W/Ni/Co and W/Ni/Fe, as well as the individual metals, in a second rodent species.
- Assessment of urine and serum metal levels as sensitive indicators of exposure to tungsten alloys and development of early detection methods for potentially exposed personnel.
- Determination of the mechanism of WA-induced tumor formation so that exposure risk can be assessed and potential treatments can be designed.

- Modeling of the deposition of the various tungsten alloy component metals in the body.
- Assessment of long-term exposure to DU on the kidney, especially the types of chronic exposure that result from embedded fragments.

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Manuscript 2 NATO RTG-099

Figure 1

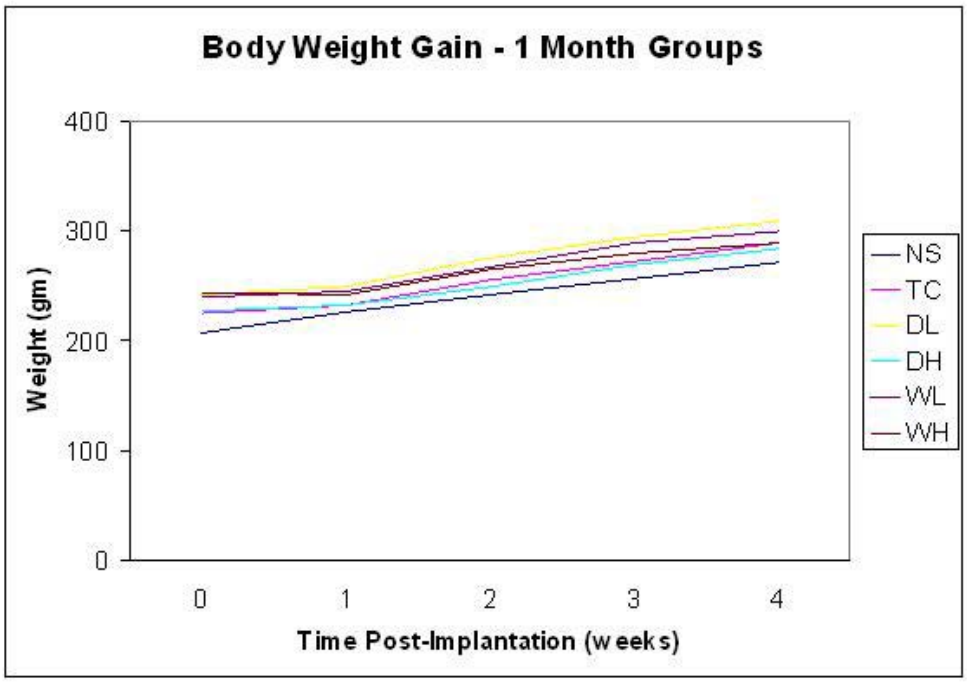

Body Weight Gain - 1 Month Groups
Weight (gm)
400
300
200
100
0
0
1
2
3
4
Time Post-Implantation (weeks)
NS
TC
DL
DH
WL
WH

Figure 2

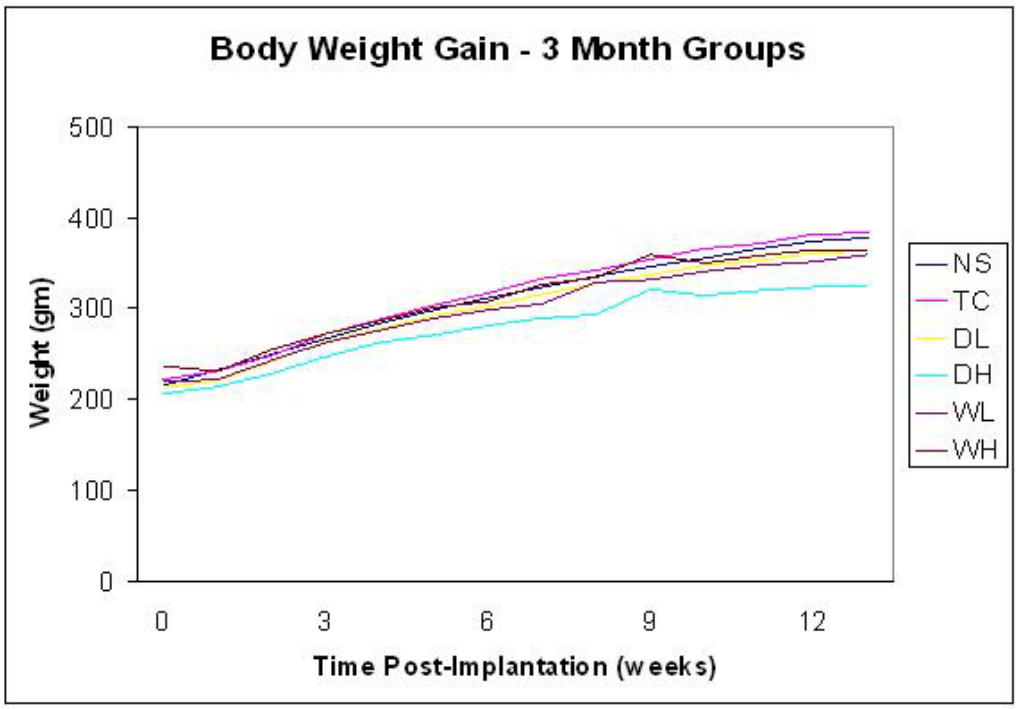

Body Weight Gain - 3 Month Groups
Weight (gm)
500
400
300
200
100
0
0
3
6
9
12
Time Post-Implantation (weeks)
NS
TC
DL
DH
WL
WH

Figure 3

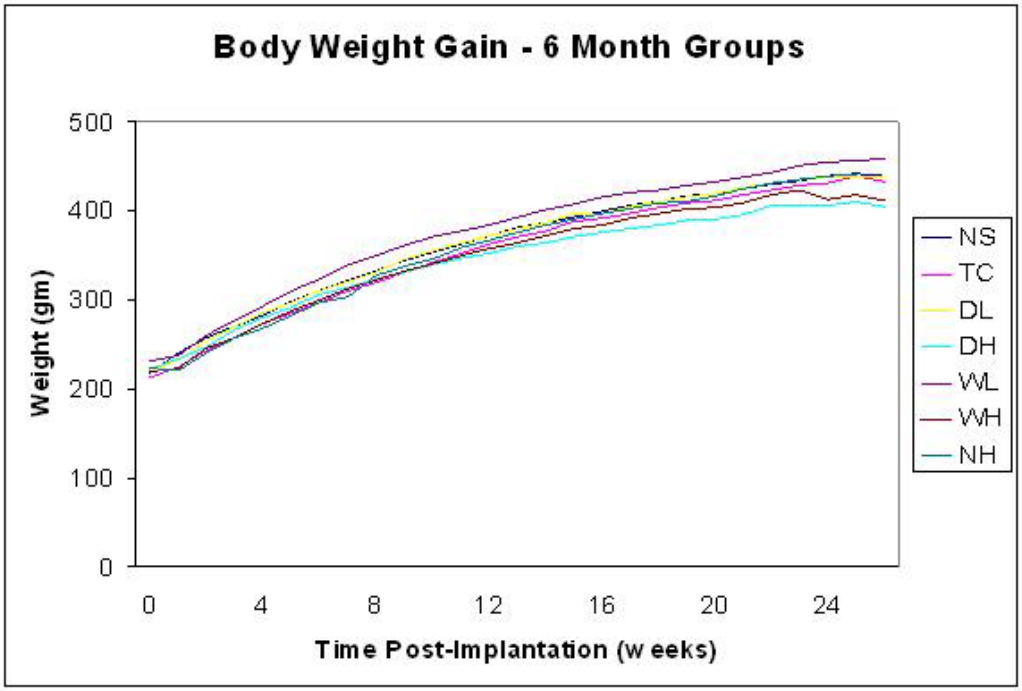

Body Weight Gain - 6 Month Groups
Weight (gm)
500
400
300
200
100
0
0
4
8
12
16
20
24
Time Post-Implantation (w eeks)
NS
TC
DL
DH
WL
WH
NH

Figure 4

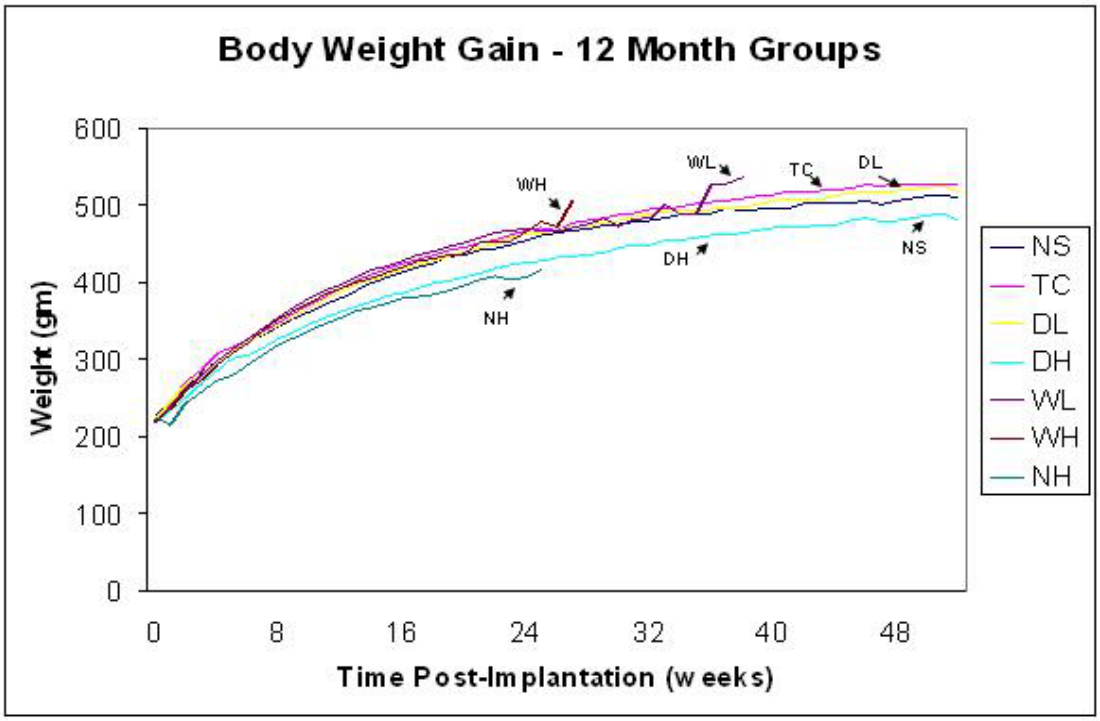

Body Weight Gain - 12 Month Groups
Weight (gm)
600
500
400
300
200
100
0
0
8
16
24
32
40
48
Time Post-Implantation (weeks)
WH
WL
TC
DL
DH
NH
NS
NS
TC
DL
DH
WL
WH
NH

Figure 5

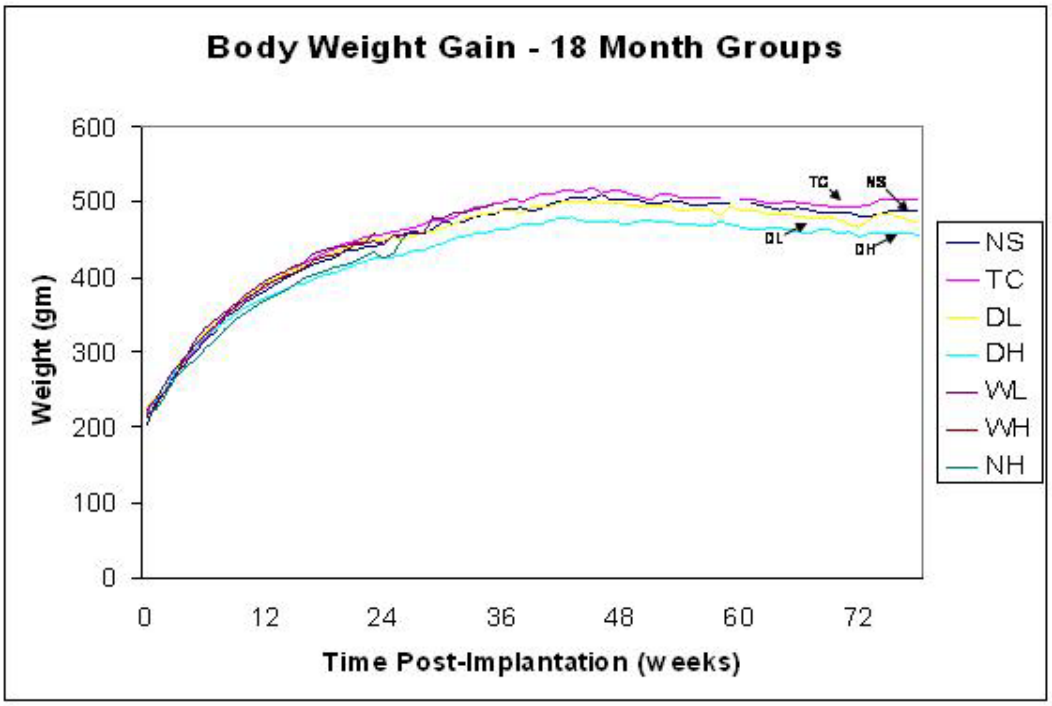

Body Weight Gain - 18 Month Groups
Weight (gm)
600
500
400
300
200
100
0
0
12
24
36
48
60
72
Time Post-Implantation (weeks)
TC
NS
DL
DH
NS
TC
DL
DH
WL
WH
NH

Figure 6

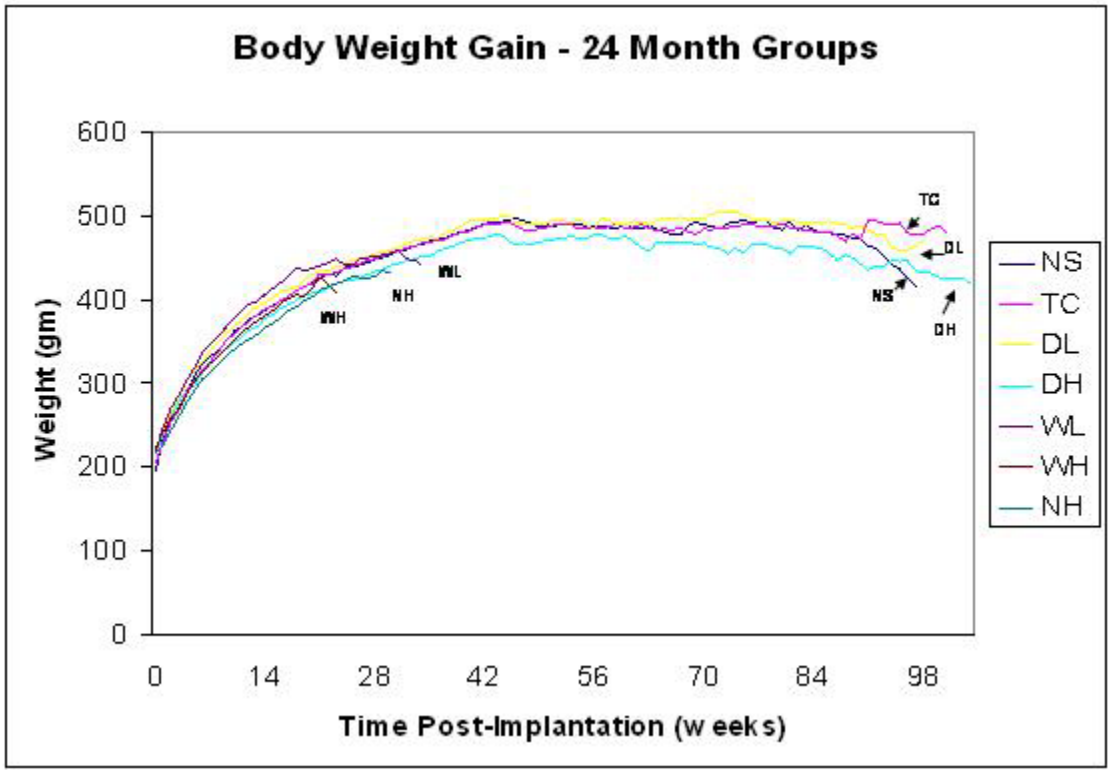

Body Weight Gain - 24 Month Groups
Weight (gm)
600
500
400
300
200
100
0
0
14
28
42
56
70
84
98
Time Post-Implantation (w eeks)
NS
TC
DL
DH
VVL
VVH
NH

Table 1. Survival Times of Pellet-Implanted Rodents

Time	Experimental Group	Mean (weeks) ± sem
24 Month	Non-Surgical	83.44 ± 2.05
(104 weeks)	Tantalum	82.69 ± 4.11
	DU-low	85.44 ± 2.82
	DU-high	81.69 ± 3.25
	WA-low	27.13 ± 1.06
	WA-high	21.75 ± 0.37
	Nickel	26.75 ± 0.59
18 Month	Non-Surgical	75.80 ± 1.20
(78 weeks)	Tantalum	74.00 ± 2.70
	DU-low	72.90 ± 2.46
	DU-high	74.30 ± 2.47
	WA-low	25.50 ± 1.83
	WA-high	20.90 ± 0.43
	Nickel	24.80 ± 0.36
12 Month	Non-Surgical	50.70 ± 0.91
(52 weeks)	Tantalum	52.00 ± 0.00
	DU-low	52.00 ± 0.00
	DU-high	52.00 ± 0.00
	WA-low	27.70 ± 0.97
	WA-high	22.25 ± 0.59
	Nickel	23.90 ± 0.31
6 Month	Non-Surgical	26.00 ± 0.00
(26 weeks)	Tantalum	26.00 ± 0.00
	DU-low	26.00 ± 0.00
	DU-high	26.00 ± 0.00
	WA-low	24.85 ± 0.52
	WA-high	22.40 ± 0.53
	Nickel	23.80 ± 0.55
3 Month	Non-Surgical	13.00 ± 0.00
(13 weeks)	Tantalum	13.00 ± 0.00
	DU-low	13.00 ± 0.00
	DU-high	13.00 ± 0.00
	WA-low	13.00 ± 0.00
	WA-high	13.00 ± 0.00
	Nickel	13.00 ± 0.00
1 Month	Non-Surgical	4.00 ± 0.00
(4 weeks)	Tantalum	4.00 ± 0.00
	DU-low	4.00 ± 0.00
	DU-high	4.00 ± 0.00
	WA-low	4.00 ± 0.00
	WA-high	4.00 ± 0.00

Table 2. Hematology Parameters for 1 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High
WBC	3.6 ± 0.2	3.9 ± 0.2	4.5 ± 0.1	3.9 ± 0.1	3.8 ± 0.1	3.9 ± 0.2
RBC	7.5 ± 0.1	7.8 ± 0.1	7.9 ± 0.1	7.6 ± 0.1	7.7 ± 0.1	8.5 ± 0.1
HGB	13.7 ± 0.1	13.7 ± 0.2	14.1 ± 0.1	13.7 ± 0.2	14.8 ± 0.2	15.8 ± 0.1
HCT	37.9 ± 0.4	40.2 ± 0.4	40.2 ± 0.5	38.4 ± 0.5	39.7 ± 0.5	43.3 ± 0.4
MCV	50.8 ± 0.1	51.2 ± 0.1	50.7 ± 0.1	50.8 ± 0.2	51.2 ± 0.3	51.0 ± 0.2
MCH	18.4 ± 0.1	17.4 ± 0.1	17.9 ± 0.2	18.1 ± 0.1	19.1 ± 0.1	18.6 ± 0.2
MCHC	36.2 ± 0.2	34.0 ± 0.1	35.3 ± 0.4	35.6 ± 0.3	37.4 ± 0.3	36.6 ± 0.4
CHCM	32.7 ± 0.1	32.2 ± 0.1	33.0 ± 0.1	33.3 ± 0.3	33.4 ± 0.3	34.1 ± 0.1
CH	16.6 ± 0.1	16.5 ± 0.0	16.7 ± 0.1	16.9 ± 0.1	17.1 ± 0.1	17.3 ± 0.0
RDW	12.1 ± 0.3	12.2 ± 0.1	12.0 ± 0.1	12.5 ± 0.2	12.7 ± 0.1	14.2 ± 0.2
HDW	2.7 ± 0.1	2.3 ± 0.0	2.4 ± 0.1	2.5 ± 0.0	2.6 ± 0.0	2.8 ± 0.0
PLT	648.3 ± 52.6	646.5 ± 18.8	653.6 ± 11.9	735.4 ± 19.8	641.0 ± 18.0	756.2 ± 43.5
MPV	10.0 ± 1.6	7.9 ± 0.4	8.1 ± 0.3	8.6 ± 0.4	8.6 ± 0.4	9.9 ± 0.5

WBC (x10e6/ul):white blood cells; RBC (x10e6/ul):red blood cells; HGB (g/dL):hemoglobin; HCT (%):hematocrit; MCV (fL):mean corpuscular volume; MCH (pg):mean corpuscular hemoglobin; MCHC (g/dL):mean corpuscular hemoglobin concentration; CHCM (g/dL):cell hemoglobin concentration mean; CH (pg):cell hemoglobin; RDW (%): red cell distribution width; HDW (g/dL):hemoglobin distribution width; PLT (x10e3/ul):platelets; MPV (fL):mean platelet volume. Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 3. Cellular Hematology for 1 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High
% Neut	20.1 ± 0.9	17.0 ± 0.9	16.9 ± 0.7	19.4 ± 0.9	21.0 ± 1.1	21.1 ± 0.9
%Lymph	74.8 ± 0.9	78.6 ± 1.1	78.9 ± 0.7	76.0 ± 1.0	74.7 ± 1.2	75.0 ± 0.9
%Mono	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.8 ± 0.1
%Eos	2.5 ± 0.2	1.8 ± 0.2	1.3 ± 0.1	2.2 ± 0.2	2.1 ± 0.2	1.2 ± 0.1
%Baso	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.4 ± 0.1	0.2 ± 0.0
%Luc	0.3 ± 0.0	0.5 ± 0.1	0.8 ± 0.1	0.4 ± 0.0	0.5 ± 0.1	0.8 ± 0.1
#Neut	0.7 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.8 ± 0.0
#Lymph	2.7 ± 0.1	3.0 ± 0.2	3.5 ± 0.1	3.0 ± 0.1	2.9 ± 0.1	2.9 ± 0.2
#Mono	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
#Eos	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
#Baso	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
#Luc	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

%Neut:percent neutrophils; %Lymph:percent lymphocytes; %Mono:percent monocytes; %Eos:percent eosinophils; %Baso:percent basophils; %Luc:percent leucocytes; # Neut:neutrophils (x10e3/ul); # Lymph:lymphocytes (x10e3/ul); # Mono:monocytes (x10e3/ul); # Eos:eosinophils (x10e3/ul); # Baso:basophils (x10e3/ul); # Luc:leucocytes (x10e3/ul). Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 4. Hematology Parameters for 3 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High
WBC	3.4 ± 0.2	2.9 ± 0.2	1.4 ± 0.7	2.8 ± 0.3	4.1 ± 0.1	4.0 ± 0.2
RBC	7.5 ± 0.2	7.5 ± 0.1	7.0 ± 0.2	7.5 ± 0.2	8.5 ± 0.2	9.1 ± 0.7
HGB	12.5 ± 0.4	12.9 ± 0.1	12.3 ± 0.4	13.7 ± 0.3	15.5 ± 0.4	17.3 ± 0.1
HCT	37.4 ± 1.2	38.1 ± 0.3	35.1 ± 1.1	37.9 ± 0.8	42.1 ± 0.7	44.8 ± 3.5
MCV	49.8 ± 0.2	51.0 ± 0.4	50.0 ± 0.3	50.4 ± 0.2	49.7 ± 0.1	48.9 ± 0.4
MCH	16.7 ± 0.2	17.3 ± 0.1	17.5 ± 0.1	18.2 ± 0.1	18.3 ± 0.2	17.7 ± 0.1
MCHC	33.5 ± 0.3	33.8 ± 0.4	34.9 ± 0.1	36.0 ± 0.3	36.7 ± 0.3	35.9 ± 0.3
CHCM	32.7 ± 0.2	31.8 ± 0.2	32.3 ± 0.2	33.2 ± 0.2	33.9 ± 0.1	33.1 ± 0.7
CH	16.3 ± 0.0	16.2 ± 0.1	16.2 ± 0.0	16.7 ± 0.1	16.8 ± 0.1	16.1 ± 0.4
RDW	12.9 ± 0.2	12.8 ± 0.3	13.1 ± 0.1	12.6 ± 0.4	12.7 ± 0.1	13.6 ± 0.1
HDW	2.4 ± 0.1	2.4 ± 0.2	2.4 ± 0.1	2.4 ± 0.1	2.4 ± 0.0	2.7 ± 0.0
PLT	470.8 ± 47.7	513.2 ± 38.4	496.4 ± 33.7	647.1 ± 40.6	585.1 ± 35.9	568.3 ± 7.5
MPV	8.8 ± 1.8	9.6 ± 1.1	10.9 ± 1.4	8.7 ± 0.4	9.1 ± 0.6	11.7 ± 0.5

WBC (x10e6/ul):white blood cells; RBC (x10e6/ul):red blood cells; HGB (g/dL):hemoglobin; HCT (%):hematocrit; MCV (fL):mean corpuscular volume; MCH (pg):mean corpuscular hemoglobin; MCHC (g/dL):mean corpuscular hemoglobin concentration; CHCM (g/dL):cell hemoglobin concentration mean; CH (pg):cell hemoglobin; RDW (%): red cell distribution width; HDW (g/dL):hemoglobin distribution width; PLT (x10e3/ul):platelets; MPV (fL):mean platelet volume. Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 5. Cellular Hematology for 3 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High
% Neut	20.3 ± 2.0	21.9 ± 1.1	25.5 ± 2.3	22.5 ± 2.0	19.7 ± 0.9	23.2 ± 1.6
%Lymph	69.5 ± 2.3	72.8 ± 1.2	59.2 ± 2.7	70.6 ± 3.0	75.3 ± 1.1	71.2 ± 1.7
%Mono	2.7 ± 1.2	1.5 ± 0.2	2.1 ± 0.3	1.9 ± 0.2	1.7 ± 0.1	2.0 ± 0.2
%Eos	7.9 ± 1.8	3.2 ± 0.3	11.0 ± 1.4	3.4 ± 1.0	2.2 ± 0.2	2.3 ± 0.2
%Baso	0.6 ± 0.1	0.5 ± 0.2	2.0 ± 0.5	0.7 ± 0.1	0.4 ± 0.1	0.6 ± 0.1
%Luc	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.7 ± 0.1
#Neut	0.7 ± 0.1	0.6 ± 0.0	0.3 ± 0.1	0.6 ± 0.0	0.8 ± 0.0	0.9 ± 0.1
#Lymph	2.4 ± 0.2	2.1 ± 0.2	0.9 ± 0.4	2.1 ± 0.2	3.1 ± 0.1	2.8 ± 0.2
#Mono	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
#Eos	0.3 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
#Baso	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
#Luc	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

%Neut:percent neutrophils; %Lymph:percent lymphocytes; %Mono:percent monocytes; %Eos:percent eosinophils; %Baso:percent basophils; %Luc:percent leucocytes; # Neut:neutrophils (x10e3/ul); # Lymph:lymphocytes (x10e3/ul); # Mono:monocytes (x10e3/ul); # Eos:eosinophils (x10e3/ul); # Baso:basophils (x10e3/ul); # Luc:leucocytes (x10e3/ul). Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 6. Hematology Parameters for 6 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High	Nickel
WBC	3.4 ± 0.2	3.2 ± 0.2	3.3 ± 0.2	2.5 ± 0.2	3.9 ± 0.4	4.6 ± 0.3	2.6 ± 0.2
RBC	8.3 ± 0.1	8.3 ± 0.1	8.2 ± 0.1	7.8 ± 0.2	8.0 ± 0.2	10.1 ± 0.1	7.5 ± 0.1
HGB	14.3 ± 0.1	14.5 ± 0.1	14.3 ± 0.1	14.1 ± 0.4	13.9 ± 0.4	16.5 ± 0.3	12.9 ± 0.2
HCT	41.8 ± 0.5	41.8 ± 0.5	40.7 ± 0.3	38.6 ± 1.1	40.4 ± 1.0	50.2 ± 0.4	38.1 ± 0.8
MCV	50.2 ± 0.1	50.2 ± 0.2	49.9 ± 0.2	49.6 ± 0.1	50.3 ± 0.3	49.7 ± 0.2	51.1 ± 0.7
MCH	17.3 ± 0.1	17.5 ± 0.1	17.6 ± 0.1	18.0 ± 0.1	17.3 ± 0.1	16.3 ± 0.3	17.4 ± 0.1
MCHC	34.4 ± 0.2	34.8 ± 0.4	35.2 ± 0.3	36.4 ± 0.1	34.5 ± 0.3	32.8 ± 0.6	34.1 ± 0.5
CHCM	32.0 ± 0.1	32.0 ± 0.1	32.5 ± 0.1	33.0 ± 0.0	32.2 ± 0.2	32.2 ± 0.1	31.7 ± 0.5
CH	16.1 ± 0.0	16.1 ± 0.0	16.2 ± 0.0	16.4 ± 0.0	16.1 ± 0.0	16.0 ± 0.0	16.1 ± 0.1
RDW	12.9 ± 0.1	12.5 ± 0.1	12.5 ± 0.1	12.9 ± 0.4	13.1 ± 0.1	13.8 ± 0.1	13.0 ± 0.2
HDW	2.3 ± 0.0	2.2 ± 0.0	2.3 ± 0.0	2.4 ± 0.1	2.4 ± 0.0	2.5 ± 0.0	2.4 ± 0.1
PLT	559 ± 14	562 ± 15	530 ± 31	573 ± 42	542 ± 14	468 ± 18	487 ± 26
MPV	8.7 ± 0.4	9.9 ± 0.7	8.2 ± 0.5	8.1 ± 0.5	8.7 ± 0.5	10.1 ± 0.6	9.0 ± 0.5

WBC (x10e6/ul):white blood cells; RBC (x10e6/ul):red blood cells; HGB (g/dL):hemoglobin; HCT (%):hematocrit; MCV (fL):mean corpuscular volume; MCH (pg):mean corpuscular hemoglobin; MCHC (g/dL):mean corpuscular hemoglobin concentration; CHCM (g/dL):cell hemoglobin concentration mean; CH (pg):cell hemoglobin; RDW (%): red cell distribution width; HDW (g/dL):hemoglobin distribution width; PLT (x10e3/ul):platelets; MPV (fL):mean platelet volume. Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 7. Cellular Hematology for 6 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High	Nickel
% Neut	29.3 ± 2.3	25.4 ± 0.8	27.5 ± 1.0	29.2 ± 1.9	30.5 ± 2.4	29.3 ± 1.6	29.5 ± 2.1
%Lymph	64.5 ± 2.5	68.6 ± 0.9	65.7 ± 1.3	65.0 ± 1.9	63.8 ± 2.4	63.9 ± 1.9	64.7 ± 2.4
%Mono	2.1 ± 0.2	2.1 ± 0.1	2.3 ± 0.2	1.7 ± 0.1	2.1 ± 0.1	2.7 ± 0.2	2.0 ± 0.3
%Eos	2.9 ± 0.2	2.7 ± 0.2	2.9 ± 0.2	2.7 ± 0.2	2.3 ± 0.1	2.7 ± 0.2	2.7 ± 0.3
%Baso	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.6 ± 0.0	0.7 ± 0.1
%Luc	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.8 ± 0.1	0.6 ± 0.1
#Neut	1.0 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	1.3 ± 0.3	1.3 ± 0.1	0.8 ± 0.1
#Lymph	2.2 ± 0.2	2.2 ± 0.2	2.2 ± 0.1	1.6 ± 0.1	2.4 ± 0.2	2.9 ± 0.2	1.6 ± 0.1
#Mono	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
#Eos	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
#Baso	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
#Luc	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

%Neut:percent neutrophils; %Lymph:percent lymphocytes; %Mono:percent monocytes; %Eos:percent eosinophils; %Baso:percent basophils; %Luc:percent leucocytes; # Neut:neutrophils (x10e3/ul); # Lymph:lymphocytes (x10e3/ul); # Mono:monocytes (x10e3/ul); # Eos:eosinophils (x10e3/ul); # Baso:basophils (x10e3/ul); # Luc:leucocytes (x10e3/ul). Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 8. Hematology Parameters for 12 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High
WBC	2.8 ± 0.1	2.6 ± 0.1	2.6 ± 0.2	2.3 ± 0.3
RBC	7.6 ± 0.1	7.6 ± 0.0	7.6 ± 0.1	7.3 ± 0.1
HGB	13.1 ± 0.1	13.2 ± 0.1	12.6 ± 0.2	12.5 ± 0.2
HCT	38.6 ± 0.5	38.4 ± 0.2	37.9 ± 0.5	36.1 ± 0.5
MCV	50.7 ± 0.2	50.3 ± 0.1	49.8 ± 0.2	49.4 ± 0.2
MCH	17.2 ± 0.1	17.3 ± 0.0	16.5 ± 0.3	17.1 ± 0.1
MCHC	34.0 ± 0.2	34.3 ± 0.1	33.2 ± 0.6	34.6 ± 0.1
CHCM	31.5 ± 0.1	31.5 ± 0.1	31.8 ± 0.1	32.2 ± 0.1
CH	15.9 ± 0.1	15.8 ± 0.1	15.8 ± 0.1	15.9 ± 0.1
RDW	13.2 ± 0.1	13.3 ± 0.1	12.9 ± 0.1	12.7 ± 0.1
HDW	2.3 ± 0.1	2.4 ± 0.1	2.3 ± 0.1	2.3 ± 0.0
PLT	621 ± 15	584 ± 18	533 ± 26	530 ± 13
MPV	9.7 ± 0.7	9.4 ± 0.6	9.0 ± 0.5	8.8 ± 0.5

WBC (x10e6/ul):white blood cells; RBC (x10e6/ul):red blood cells; HGB (g/dL):hemoglobin; HCT (%):hematocrit; MCV (fL):mean corpuscular volume; MCH (pg):mean corpuscular hemoglobin; MCHC (g/dL):mean corpuscular hemoglobin concentration; CHCM (g/dL):cell hemoglobin concentration mean; CH (pg):cell hemoglobin; RDW (%): red cell distribution width; HDW (g/dL):hemoglobin distribution width; PLT (x10e3/ul):platelets; MPV (fL):mean platelet volume. Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 9. Cellular Hematology for 12 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High
% Neut	34.1 ± 1.6	34.8 ± 1.2	33.5 ± 1.6	38.9 ± 2.1
%Lymph	59.3 ± 1.8	57.7 ± 1.5	58.8 ± 2.0	50.6 ± 2.9
%Mono	2.5 ± 0.1	2.8 ± 0.2	2.9 ± 0.2	2.7 ± 0.2
%Eos	3.0 ± 0.3	3.7 ± 0.6	4.0 ± 0.7	6.1 ± 1.0
%Baso	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	1.4 ± 0.2
%Luc	0.3 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	0.6 ± 0.1
#Neut	0.9 ± 0.1	0.9 ± 0.0	0.9 ± 0.1	0.9 ± 0.2
#Lymph	1.6 ± 0.1	1.5 ± 0.1	1.6 ± 0.1	1.2 ± 0.2
#Mono	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
#Eos	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
#Baso	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
#Luc	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

%Neut:percent neutrophils; %Lymph:percent lymphocytes; %Mono:percent monocytes; %Eos:percent eosinophils; %Baso:percent basophils; %Luc:percent leucocytes; # Neut:neutrophils (x10e3/ul); # Lymph:lymphocytes (x10e3/ul); # Mono:monocytes (x10e3/ul); # Eos:eosinophils (x10e3/ul); # Baso:basophils (x10e3/ul); # Luc:leucocytes (x10e3/ul). Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 10. Hematology Parameters for 18 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High
WBC	2.8 ± 1.4	1.1 ± 0.4	10.6 ± 7.3	0.7 ± 0.1
RBC	7.2 ± 0.3	7.3 ± 0.3	6.5 ± 0.8	7.4 ± 0.1
HGB	13.5 ± 0.4	13.2 ± 0.7	11.8 ± 1.2	12.7 ± 0.2
HCT	37.7 ± 1.1	36.6 ± 1.7	34.8 ± 2.4	35.6 ± 0.4
MCV	50.4 ± 0.8	49.7 ± 0.4	120.5 ± 61.2	48.4 ± 0.6
MCH	18.0 ± 0.3	17.9 ± 0.2	18.7 ± 0.9	17.3 ± 0.2
MCHC	35.8 ± 0.2	36.0 ± 0.2	33.2 ± 1.5	35.8 ± 0.2
CHCM	33.5 ± 0.5	34.1 ± 0.3	33.2 ± 0.7	34.3 ± 0.3
CH	16.8 ± 0.1	16.9 ± 0.1	18.9 ± 1.5	16.5 ± 0.1
RDW	14.2 ± 0.2	13.7 ± 0.3	15.6 ± 1.1	13.3 ± 0.1
HDW	2.7 ± 0.1	2.7 ± 0.1	3.2 ± 0.3	2.7 ± 0.1
PLT	616 ± 63	605 ± 17	505 ± 53	538 ± 22
MPV	10.9 ± 2.0	7.6 ± 0.3	8.0 ± 0.5	8.4 ± 0.6

WBC (x10e6/ul):white blood cells; RBC (x10e6/ul):red blood cells; HGB (g/dL):hemoglobin; HCT (%):hematocrit; MCV (fL):mean corpuscular volume; MCH (pg):mean corpuscular hemoglobin; MCHC (g/dL):mean corpuscular hemoglobin concentration; CHCM (g/dL):cell hemoglobin concentration mean; CH (pg):cell hemoglobin; RDW (%): red cell distribution width; HDW (g/dL):hemoglobin distribution width; PLT (x10e3/ul):platelets; MPV (fL):mean platelet volume. Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table11. Cellular Hematology for 18 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High
% Neut	41.0 ± 3.6	42.6 ± 2.5	39.6 ± 8.5	48.1 ± 2.5
%Lymph	47.0 ± 3.8	45.6 ± 2.8	37.4 ± 8.6	39.9 ± 2.8
%Mono	4.0 ± 0.6	4.1 ± 0.3	4.0 ± 0.4	3.3 ± 0.2
%Eos	6.2 ± 1.5	6.4 ± 1.1	7.5 ± 2.6	6.3 ± 0.6
%Baso	1.2 ± 0.3	0.5 ± 0.1	1.5 ± 0.7	0.6 ± 0.1
%Luc	1.0 ± 0.3	0.9 ± 0.2	10.8 ± 9.5	0.4 ± 0.1
#Neut	0.8 ± 0.3	0.5 ± 0.2	1.1 ± 0.5	0.3 ± 0.0
#Lymph	1.6 ± 1.0	0.5 ± 0.2	3.8 ± 2.4	0.3 ± 0.0
#Mono	0.1 ± 0.1	0.1 ± 0.0	0.4 ± 0.3	0.0 ± 0.0
#Eos	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
#Baso	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.4	0.0 ± 0.0
#Luc	0.0 ± 0.0	0.0 ± 0.0	5.1 ± 5.0	0.0 ± 0.0

%Neut:percent neutrophils; %Lymph:percent lymphocytes; %Mono:percent monocytes; %Eos:percent eosinophils; %Baso:percent basophils; %Luc:percent leucocytes; # Neut:neutrophils (x10e3/ul); # Lymph:lymphocytes (x10e3/ul); # Mono:monocytes (x10e3/ul); # Eos:eosinophils (x10e3/ul); # Baso:basophils (x10e3/ul); # Luc:leucocytes (x10e3/ul). Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 12. Hematology Parameters for 24 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High
WBC	20.2 ± 10.2	3.0 ± 0.7	28.1 ± 25.0	11.1 ± 6.0
RBC	5.1 ± 1.0	6.9 ± 0.4	7.1 ± 0.9	6.8 ± 0.4
HGB	9.8 ± 1.4	13.6 ± 0.6	13.4 ± 1.7	12.3 ± 0.7
HCT	31.6 ± 2.9	37.0 ± 1.6	37.8 ± 4.8	35.9 ± 1.7
MCV	75.3 ± 13.8	53.9 ± 1.2	52.9 ± 1.0	52.9 ± 0.7
MCH	21.3 ± 2.1	19.9 ± 1.0	18.8 ± 0.5	18.0 ± 0.4
MCHC	30.2 ± 2.0	36.9 ± 1.3	35.5 ± 0.4	34.1 ± 0.6
CHCM	29.6 ± 1.3	31.3 ± 0.4	32.2 ± 0.9	32.1 ± 0.3
CH	21.2 ± 2.7	16.7 ± 0.2	17.1 ± 0.3	16.9 ± 0.3
RDW	17.5 ± 1.1	14.8 ± 0.4	14.7 ± 1.9	15.1 ± 1.0
HDW	3.1 ± 0.3	2.7 ± 0.1	2.3 ± 0.0	2.7 ± 0.2
PLT	523 ± 113	1063 ± 280	650 ± 129	611 ± 29
MPV	10.7 ± 1.7	13.5 ± 3.9	12.6 ± 3.3	8.1 ± 0.5

WBC (x10e6/ul):white blood cells; RBC (x10e6/ul):red blood cells; HGB (g/dL):hemoglobin; HCT (%):hematocrit; MCV (fL):mean corpuscular volume; MCH (pg):mean corpuscular hemoglobin; MCHC (g/dL):mean corpuscular hemoglobin concentration; CHCM (g/dL):cell hemoglobin concentration mean; CH (pg):cell hemoglobin; RDW (%): red cell distribution width; HDW (g/dL):hemoglobin distribution width; PLT (x10e3/ul):platelets; MPV (fL):mean platelet volume. Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 13. Cellular Hematology for 24 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High
% Neut	26.7 ± 6.2	47.0 ± 4.6	42.3 ± 5.2	54.5 ± 5.1
%Lymph	61.2 ± 6.3	43.8 ± 5.8	40.8 ± 6.9	35.4 ± 5.3
%Mono	4.2 ± 0.9	2.6 ± 0.3	9.6 ± 5.3	3.7 ± 0.6
%Eos	3.7 ± 2.3	5.2 ± 1.7	1.5 ± 0.3	4.7 ± 1.4
%Baso	1.4 ± 0.5	2.1 ± 0.6	5.1 ± 4.3	0.9 ± 0.4
%Luc	2.8 ± 1.3	0.3 ± 0.1	1.0 ± 0.3	0.9 ± 0.3
#Neut	3.1 ± 1.1	1.3 ± 0.3	7.3 ± 5.9	7.1 ± 4.0
#Lymph	14.6 ± 7.9	1.5 ± 0.5	4.5 ± 3.1	3.4 ± 1.9
#Mono	1.1 ± 0.7	0.1 ± 0.0	9.3 ± 9.2	0.4 ± 0.2
#Eos	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
#Baso	0.3 ± 0.2	0.1 ± 0.0	6.8 ± 6.7	0.0 ± 0.0
#Luc	0.9 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

%Neut:percent neutrophils; %Lymph:percent lymphocytes; %Mono:percent monocytes; %Eos:percent eosinophils; %Baso:percent basophils; %Luc:percent leucocytes; # Neut:neutrophils (x10e3/ul); # Lymph:lymphocytes (x10e3/ul); # Mono:monocytes (x10e3/ul); # Eos:eosinophils (x10e3/ul); # Baso:basophils (x10e3/ul); # Luc:leucocytes (x10e3/ul). Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 14. Serum Chemistries for 1 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High
GLU	206.8 ± 18.3	217.3 ± 8.7	232.1 ± 10.0	191.6 ± 9.7	171.1 ± 9.2	206.8 ± 7.1
BUN	13.2 ± 1.1	14.5 ± 0.5	13.9 ± 0.4	14.9 ± 0.4	13.1 ± 0.4	16.9 ± 0.3
CREA	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0
NA	170.1 ± 13.0	160.3 ± 8.1	159.9 ± 8.2	163.4 ± 9.9	140.2 ± 0.7	145.9 ± 0.7
K	4.1 ± 0.3	4.0 ± 0.1	3.9 ± 0.1	4.1 ± 0.1	3.6 ± 0.1	4.4 ± 0.1
CA	9.5 ± 0.5	9.4 ± 0.1	9.6 ± 0.1	9.2 ± 0.2	7.9 ± 0.4	10.4 ± 0.1
PHOS	5.9 ± 0.3	6.3 ± 0.2	6.7 ± 0.1	6.5 ± 0.3	5.3 ± 0.3	6.9 ± 0.2
URIC	1.4 ± 0.4	0.5 ± 0.0	0.5 ± 0.1	0.8 ± 0.1	0.6 ± 0.1	0.9 ± 0.1
Tot Pro	4.9 ± 0.3	5.1 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	4.9 ± 0.1	5.8 ± 0.1
ALB	2.2 ± 0.2	2.3 ± 0.1	2.3 ± 0.0	2.2 ± 0.1	2.0 ± 0.1	2.7 ± 0.0
LDH	2679 ± 950	3274 ± 565	3501 ± 511	3328 ± 656	2312 ± 370	2146 ± 389
ALKP	276.4 ± 25.1	289.2 ± 11.8	287.3 ± 12.4	239.1 ± 17.9	176.8 ± 10.0	260.0 ± 5.9

GLU:glucose (mg/dL); BUN:blood urea nitrogen (mg/dL); CREA:creatinine (mg/dL); NA:sodium (mmol/L);
K:potassium (mmol/L); CA:calcium (mg/dL); PHOS:phosphorus (mg/dL); URIC:uric acid (mg/dL);
Tot Pro:total protein (g/dL); ALB:albumin (g/dL); LDH;lactate dehydrogenase (U/L); ALKP:alkaline phosphatase (U/L).
Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 15. Serum Chemistries for 3 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High
GLU	241.6 ± 20.9	214.4 ± 6.8	242.9 ± 7.5	186.9 ± 7.2	215.7 ± 8.5	217.4 ± 9.9
BUN	18.4 ± 1.5	17.6 ± 0.5	18.2 ± 0.5	15.7 ± 0.3	16.3 ± 0.5	16.0 ± 0.6
CREA	0.6 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0
NA	180.9 ± 8.6	136.6 ± 0.5	142.3 ± 3.6	181.8 ± 1.9	181.9 ± 1.7	172.0 ± 5.0
K	4.2 ± 0.2	4.0 ± 0.1	4.3 ± 0.1	3.6 ± 0.1	3.7 ± 0.1	3.9 ± 0.1
CA	9.6 ± 0.7	9.6 ± 0.2	10.2 ± 0.2	9.0 ± 0.2	8.9 ± 0.2	9.7 ± 0.4
PHOS	5.2 ± 0.4	5.3 ± 0.2	6.1 ± 0.2	4.3 ± 0.1	4.7 ± 0.3	5.1 ± 0.3
URIC	1.0 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1	0.6 ± 0.1	0.8 ± 0.1
Tot Pro	5.4 ± 0.3	5.6 ± 0.1	5.8 ± 0.1	5.0 ± 0.1	5.5 ± 0.1	5.6 ± 0.2
ALB	2.6 ± 0.2	2.7 ± 0.1	2.9 ± 0.1	2.1 ± 0.1	2.1 ± 0.1	2.5 ± 0.2
LDH	2719 ± 462	2933 ± 372	2536 ± 358	2110 ± 343	2318 ± 285	2519 ± 273
ALKP	180.9 ± 17.0	200.0 ± 7.2	200.8 ± 8.6	148.9 ± 4.9	163.7 ± 7.9	163.0 ± 11.7

GLU:glucose (mg/dL); BUN:blood urea nitrogen (mg/dL); CREA:creatinine (mg/dL); NA:sodium (mmol/L);
K:potassium (mmol/L); CA:calcium (mg/dL); PHOS:phosphorus (mg/dL); URIC:uric acid (mg/dL);
Tot Pro:total protein (g/dL); ALB:albumin (g/dL); LDH;lactate dehydrogenase (U/L); ALKP:alkaline phosphatase (U/L).
Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 16. Serum Chemistries for 6 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High	Nickel
GLU	229.4 ± 7.1	245.8 ± 9.2	246.7 ± 6.6	241.6 ± 3.3	246.6 ± 4.1	230.5 ± 4.3	248.1 ± 8.3
BUN	16.5 ± 0.3	16.5 ± 0.3	16.1 ± 0.5	15.9 ± 0.7	14.5 ± 0.6	13.5 ± 0.5	12.9 ± 0.3
CREA	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.4 ± 0.0
NA	138.0 ± 0.3	139.1 ± 0.7	137.8 ± 0.3	138.6 ± 0.3	137.9 ± 0.4	138.3 ± 0.3	138.9 ± 0.6
K	4.7 ± 0.2	4.7 ± 0.2	4.7 ± 0.1	4.3 ± 0.1	4.2 ± 0.1	4.1 ± 0.1	4.0 ± 0.1
CA	10.1 ± 0.0	10.0 ± 0.1	9.9 ± 0.0	10.0 ± 0.1	10.0 ± 0.1	9.9 ± 0.0	10.1 ± 0.1
PHOS	5.4 ± 0.2	4.9 ± 0.2	5.3 ± 0.2	5.4 ± 0.2	5.1 ± 0.1	5.0 ± 0.2	5.2 ± 0.2
URIC	1.2 ± 0.1	0.9 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
Tot Pro	6.2 ± 0.1	6.2 ± 0.1	6.1 ± 0.1	5.8 ± 0.1	5.7 ± 0.0	5.7 ± 0.1	5.6 ± 0.0
ALB	2.9 ± 0.0	2.9 ± 0.1	2.8 ± 0.1	2.6 ± 0.1	2.6 ± 0.0	2.5 ± 0.0	2.5 ± 0.0
LDH	6876 ± 585	6654 ± 543	6706 ± 629	5253 ± 806	4727 ± 452	4241 ± 454	3165 ± 498
ALKP	216.2 ± 5.8	223.6 ± 4.9	199.7 ± 7.9	207.0 ± 10.3	212.6 ± 8.9	190.3 ± 5.3	229.5 ± 10.7

GLU:glucose (mg/dL); BUN:blood urea nitrogen (mg/dL); CREA:creatinine (mg/dL); NA:sodium (mmol/L);
K:potassium (mmol/L); CA:calcium (mg/dL); PHOS:phosphorus (mg/dL); URIC:uric acid (mg/dL);
Tot Pro:total protein (g/dL); ALB:albumin (g/dL); LDH;lactate dehydrogenase (U/L); ALKP:alkaline phosphatase (U/L).
Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 17. Serum Chemistries for 12 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High
GLU	247.1 ± 6.1	232.1 ± 5.2	213.2 ± 6.2	220.6 ± 10.7
BUN	15.4 ± 0.6	16.0 ± 0.7	15.7 ± 0.8	17.1 ± 1.1
CREA	0.5 ± 0.0	0.5 ± 0.0	0.5 ± 0.0	0.6 ± 0.0
NA	142.7 ± 0.9	146.0 ± 2.4	146.7 ± 4.3	169.6 ± 7.1
K	4.2 ± 0.0	4.3 ± 0.1	4.5 ± 0.1	4.2 ± 0.1
CA	10.2 ± 0.1	10.4 ± 0.2	10.0 ± 0.2	9.9 ± 0.4
PHOS	4.9 ± 0.1	4.6 ± 0.2	4.7 ± 0.1	4.5 ± 0.3
URIC	0.8 ± 0.1	0.7 ± 0.0	0.8 ± 0.1	0.7 ± 0.1
Tot Pro	5.8 ± 0.1	6.0 ± 0.1	5.8 ± 0.2	6.0 ± 0.2
ALB	2.8 ± 0.0	3.0 ± 0.1	2.8 ± 0.1	2.7 ± 0.2
LDH	4321 ± 512	5044 ± 548	4456 ± 622	3818 ± 536
ALKP	178.8 ± 7.6	194.6 ± 7.4	174.4 ± 9.0	171.0 ± 10.7

GLU:glucose (mg/dL); BUN:blood urea nitrogen (mg/dL); CREA:creatinine (mg/dL); NA:sodium (mmol/L);
K:potassium (mmol/L); CA:calcium (mg/dL); PHOS:phosphorus (mg/dL); URIC:uric acid (mg/dL);
Tot Pro:total protein (g/dL); ALB:albumin (g/dL); LDH;lactate dehydrogenase (U/L); ALKP:alkaline phosphatase (U/L).
Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 18. Serum Chemistries for 18 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High
GLU	162.1 ± 12.4	157.4 ± 14.8	155.8 ± 13.7	177.6 ± 12.5
BUN	15.9 ± 0.9	15.1 ± 1.5	13.6 ± 0.7	15.8 ± 0.9
CREA	0.6 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.6 ± 0.0
NA	157.1 ± 10.3	165.8 ± 11.1	168.9 ± 10.7	157.3 ± 9.0
K	4.9 ± 0.2	5.0 ± 0.3	4.4 ± 0.2	4.5 ± 0.2
CA	10.5 ± 0.5	10.1 ± 0.8	9.7 ± 0.2	10.6 ± 0.2
PHOS	4.7 ± 0.3	4.3 ± 0.5	4.2 ± 0.3	4.5 ± 0.3
URIC	0.8 ± 0.1	0.6 ± 0.1	0.9 ± 0.2	0.6 ± 0.1
Tot Pro	6.7 ± 0.3	6.3 ± 0.6	5.8 ± 0.2	6.6 ± 0.3
ALB	3.0 ± 0.1	2.9 ± 0.3	2.5 ± 0.1	3.1 ± 0.1
LDH	4737 ± 406	3258 ± 693	4162 ± 585	3277 ± 595
ALKP	211.0 ± 20.9	174.7 ± 24.0	191.1 ± 20.1	190.3 ± 5.8

GLU:glucose (mg/dL); BUN:blood urea nitrogen (mg/dL); CREA:creatinine (mg/dL); NA:sodium (mmol/L);
K:potassium (mmol/L); CA:calcium (mg/dL); PHOS:phosphorus (mg/dL); URIC:uric acid (mg/dL);
Tot Pro:total protein (g/dL); ALB:albumin (g/dL); LDH;lactate dehydrogenase (U/L); ALKP:alkaline phosphatase (U/L).
Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 19. Serum Chemistries for 24 Month Rodents

	Non-Surgical	Tantalum	DU-Low	DU-High
GLU	148.5 ± 15.8	179.7 ± 16.1	181.7 ± 20.5	192.9 ± 12.0
BUN	16.8 ± 1.5	19.1 ± 0.9	18.5 ± 1.2	18.6 ± 1.2
CREA	0.6 ± 0.0	0.7 ± 0.1	0.6 ± 0.0	0.7 ± 0.1
NA	139.8 ± 2.5	146.6 ± 2.0	156.3 ± 3.3	146.7 ± 5.1
K	4.3 ± 0.2	4.6 ± 0.1	4.6 ± 0.2	4.1 ± 0.1
CA	10.0 ± 0.3	10.6 ± 0.1	11.1 ± 0.2	10.6 ± 0.3
PHOS	5.1 ± 0.3	4.8 ± 0.2	5.5 ± 0.2	5.0 ± 0.3
URIC	1.0 ± 0.1	1.1 ± 0.1	0.7 ± 0.1	0.8 ± 0.2
Tot Pro	5.5 ± 0.1	6.1 ± 0.1	6.4 ± 0.2	5.9 ± 0.2
ALB	2.6 ± 0.1	2.9 ± 0.1	3.3 ± 0.1	2.9 ± 0.2
LDH	3566 ± 659	3328 ± 626	3325 ± 639	1987 ± 579
ALKP	172.8 ± 22.4	181.1 ± 19.0	177.2 ± 22.2	204.3 ± 23.1

GLU:glucose (mg/dL); BUN:blood urea nitrogen (mg/dL); CREA:creatinine (mg/dL); NA:sodium (mmol/L);
K:potassium (mmol/L); CA:calcium (mg/dL); PHOS:phosphorus (mg/dL); URIC:uric acid (mg/dL);
Tot Pro:total protein (g/dL); ALB:albumin (g/dL); LDH;lactate dehydrogenase (U/L); ALKP:alkaline phosphatase (U/L).
Data represent the mean of 15 observations. Errors are given as standard error of the mean.

Table 20. Tumor Distribution in Experimental Animals Based on Gross Necropsy Examination.

Time / Group	1 Month (n = 15)	3 Month (n = 15)	6 Month (n = 20)	12 Month (n = 20)	18 Month (n = 10)	24 Month (n = 16)
Non-surgical	None	None	None	1-abdominal	8-testicle	7-testicle 3-abdominal
Tantalum	None	None	2-abdominal	None	8-testicle 2-abdominal	9-testicle 5-abdominal 1-muscle (leg)
DU Low Dose	None	None	None	2-abdominal	8-testicle 1-abdominal	10-testicle 1-abdominal 1-adrenal
DU High Dose	None	None	None	2-abdominal 1-lung	6-testicle 2-abdominal 1-adrenal	9-testicle, 2-lung 5-kidney, 1-muscle (leg)
WA Low Dose	None	None	20-muscle (leg) 2-lung	20-muscle (leg) 2-lung 1-abdominal	10-muscle (leg) 4-lung	16-muscle (leg) 6-lung 2-abdominal
WA High Dose	None	None	20-muscle (leg) 3-lung	20-muscle (leg) 2-lung 1-abdominal	10-muscle (leg) 4-lung 1-abdominal	16-muscle (leg) 6-lung
Nickel	ND	ND	10-muscle (leg) (n = 10)	10-muscle (leg) 1-abdominal (n = 10)	10-muscle (leg) (n = 10)	16-muscle (leg) (n = 16)

Table 21. Organ Weights and Organ/Body Weight Ratios for 1-Month Rodents

1 Month	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High
Spleen (mg)	652 ± 10	682 ± 13	710 ± 9 *	666 ± 20	725 ± 15 *	790 ± 14 *
Thymus (mg)	316 ± 11	308 ± 11	359 ± 16	309 ± 15	342 ± 13	307 ± 10
Liver (gm)	9.1 ± 0.2	10.0 ± 0.2	10.6 ± 0.2 *	9.7 ± 0.3	10.3 ± 0.1 *	9.9 ± 0.3 *
Kidney (mg)	806 ± 17	892 ± 18	957 ± 17	846 ± 19	909 ± 12 *	856 ± 15 *
Testes (mg)	1393 ± 21	1457 ± 19	1520 ± 15 *	1437 ± 23	1478 ± 15 *	1442 ± 19
Spleen/BW	2.4 ± 0.0	2.4 ± 0.1	2.3 ± 0.0	2.4 ± 0.0	2.4 ± 0.0	2.7 ± 0.0 *
Thymus/BW	1.2 ± 0.0	1.1 ± 0.0	1.2 ± 0.0	1.1 ± 0.0	1.1 ± 0.0	1.1 ± 0.0
Liver/BW	33.6 ± 0.2	34.5 ± 0.3	34.2 ± 0.2	34.2 ± 0.4	34.3 ± 0.2 *	34.2 ± 0.6
Kidney/BW	5.9 ± 0.1	6.2 ± 0.1	6.2 ± 0.1 *	6.0 ± 0.1	6.1 ± 0.1	5.9 ± 0.1
Testes/BW	10.3 ± 0.1	10.1 ± 0.2	9.9 ± 0.1 *	10.2 ± 0.1	9.9 ± 0.1 *	10.0 ± 0.1

Data are expressed as mean +/- standard error of the mean of 15 replicates. * represents a significant difference from control at $P<0.05$ using Students t-test.

Table 22. Organ Weights and Organ/Body Weight Ratios for 3-Month Rodents

3 Month	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High
Spleen (mg)	775 ± 10	795 ± 21	774 ± 14	719 ± 24 *	774 ± 13	909 ± 21 *
Thymus (mg)	278 ± 11	281 ± 13	249 ± 8	215 ± 16 *	303 ± 9	252 ± 13
Liver (gm)	11.5 ± 0.2	11.7 ± 0.2	11.2 ± 0.2	9.6 ± 0.2 *	11.2 ± 0.3	11.2 ± 0.3
Kidney (mg)	998 ± 17	1041 ± 14	1025 ± 23	883 ± 24 *	1029 ± 37	1047 ± 26
Testes (mg)	1536 ± 25	1599 ± 19	1539 ± 26	1474 ± 29	1484 ± 97	1525 ± 36
Spleen/BW	2.1 ± 0.0	2.1 ± 0.0	2.1 ± 0.0	2.2 ± 0.1	2.2 ± 0.0 *	2.5 ± 0.0 *
Thymus/BW	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.8 ± 0.0 *	0.7 ± 0.0
Liver/BW	30.4 ± 0.2	30.6 ± 0.3	30.8 ± 0.3	29.7 ± 0.3 *	31.0 ± 0.3	30.3 ± 0.3
Kidney/BW	5.3 ± 0.0	5.4 ± 0.1	5.6 ± 0.1 *	5.4 ± 0.1	5.7 ± 0.2	5.8 ± 0.0 *
Testes/BW	8.1 ± 0.1	8.3 ± 0.1	8.4 ± 0.1	9.1 ± 0.1 *	8.2 ± 0.5	8.4 ± 0.2

Data are expressed as mean +/- standard error of the mean of 15 replicates. * represents a significant difference from control at $P<0.05$ using Students t-test.

Table 23. Organ Weights and Organ/Body Weight Ratios for 6-Month Rodents

6 Month	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High	Nickel
Spleen (mg)	938 ± 29	936 ± 39	959 ± 29	862 ± 14 *	1010 ± 28	1042 ± 16 *	901 ± 18
Thymus (mg)	378 ± 34	374 ± 17	423 ± 20	299 ± 23	335 ± 12	315 ± 14	274 ± 15 *
Liver (gm)	12.9 ± 0.2	12.6 ± 0.3	12.8 ± 0.2	11.5 ± 0.2 *	13.9 ± 0.2	12.5 ± 0.2	12.4 ± 0.2
Kidney (mg)	1128 ± 15	1111 ± 25	1144 ± 32	1007 ± 15 *	1220 ± 14 *	1130 ± 11	1112 ± 15
Testes (mg)	1548 ± 25	1579 ± 28	1545 ± 32	1557 ± 28	1610 ± 17 *	1552 ± 14	1544 ± 31
Spleen/BW	2.1 ± 0.1	2.2 ± 0.1	2.2 ± 0.1	2.1 ± 0.0	2.2 ± 0.1	2.74± 0.0 *	2.1 ± 0.0
Thymus/BW	0.8 ± 0.1	0.9 ± 0.0	1.0 ± 0.0	0.7 ± 0.1	0.7 ± 0.0	0.8 ± 0.1	0.6 ± 0.0 *
Liver/BW	29.3 ± 0.3	29.2 ± 0.3	29.1 ± 0.2	28.3 ± 0.3 *	30.0 ± 0.2	29.1 ± 0.2	28.9 ± 0.3
Kidney/BW	5.1 ± 0.1	5.1 ± 0.1	5.2 ± 0.1	5.0 ± 0.1 *	5.3 ± 0.1 *	5.3 ± 0.1 *	5.2 ± 0.1
Testes/BW	7.0 ± 0.1	7.3 ± 0.1	7.0 ± 0.1	7.7 ± 0.1 *	7.0 ± 0.1	7.3 ± 0.1 *	7.2 ± 0.1

Data are expressed as mean +/- standard error of the mean of 20 replicates. * represents a significant difference from control at $P<0.05$ using Students t-test.

Table 24. Organ Weights and Organ/Body Weight Ratios for 12-Month Rodents

12 Month	Non-Surgical	Tantalum	DU-Low	DU-High
Spleen (mg)	1045 ± 22	990 ± 25	1015 ± 21	976 ± 24 *
Thymus (mg)	347 ± 34	334 ± 24	301 ± 21	284 ± 22
Liver (gm)	14.4 ± 0.3	15.2 ± 0.3	14.9 ± 0.2	13.5 ± 0.2 *
Kidney (mg)	1303 ± 19	1306 ± 24	1313 ± 17	1210 ± 18 *
Testes (mg)	1660 ± 23	1708 ± 19	1746 ± 28	1659 ± 19
Spleen/BW	2.1 ± 0.2	1.9 ± 0.1	1.9 ± 0.1	2.0 ± 0.2
Thymus/BW	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.0
Liver/BW	28.3 ± 0.4	29.3 ± 0.2	28.7 ± 0.3	28.0 ± 0.3
Kidney/BW	5.1 ± 0.1	5.1 ± 0.1	5.1 ± 0.1	5.0 ± 0.1
Testes/BW	6.5 ± 0.1	6.6 ± 0.1	6.7 ± 0.1	6.9 ± 0.1 *

Data are expressed as mean +/- standard error of the mean of 10 replicates. * represents a significant difference from control at $P<0.05$ using Students t-test.

Table 25. Organ Weights and Organ/Body Weight Ratios for 18-Month Rodents

18 Month	Non-Surgical	Tantalum	DU-Low	DU-High
Spleen (mg)	1701 ± 401	1242 ± 88	3119 ± 1431	1094 ± 35
Thymus (mg)	ND	ND	ND	ND
Liver (gm)	14.4 ± 0.3	14.4 ± 0.4	14.1 ± 0.6	12.8 ± 0.4 *
Kidney (mg)	1374 ± 23	1425 ± 19	1383 ± 25	1288 ± 26 *
Testes (mg)	1734 ± 70	1724 ± 73	1656 ± 75	1668 ± 90
Spleen/BW	3.6 ± 0.9	2.5 ± 0.2	6.9 ± 3.2	2.4 ± 0.1
Thymus/BW	ND	ND	ND	ND
Liver/BW	30.0 ± 0.8	29.2 ± 0.8	30.2 ± 1.2	27.4 ± 0.6 *
Kidney/BW	5.7 ± 0.1	5.8 ± 0.1	5.9 ± 0.1	5.5 ± 0.1
Testes/BW	7.2 ± 0.3	7.0 ± 0.3	7.1 ± 0.3	7.2 ± 0.4

Data are expressed as mean +/- standard error of the mean of 10 replicates. * represents a significant difference from control at $P<0.05$ using Students t-test. ND is not determined.

Table 26. Organ Weights and Organ/Body Weight Ratios for 24-Month Rodents

24 Month	Non-Surgical	Tantalum	DU-Low	DU-High
Spleen (mg)	4400 ± 1609	1612 ± 337	2608 ± 1219	1488 ± 174
Thymus (mg)	ND	ND	ND	ND
Liver (gm)	15.2 ± 1.2	14.7 ± 0.8	15.3 ± 2.1	13.2 ± 0.9
Kidney (mg)	1494 ± 41	1410 ± 67	1519 ± 38	1463 ± 94
Testes (mg)	1480 ± 151	1549 ± 119	1505 ± 130	1455 ± 90
Spleen/BW	10.2 ± 3.6	3.5 ± 0.7	5.5 ± 2.6	3.5 ± 0.4
Thymus/BW	ND	ND	ND	ND
Liver/BW	33.8 ± 2.6	31.7 ± 1.4	32.9 ± 4.4	30.5 ± 2.3
Kidney/BW	6.7 ± 0.3	6.1 ± 0.3	6.6 ± 0.1	6.8 ± 0.5
Testes/BW	6.5 ± 0.6	6.8 ± 0.5	6.5 ± 0.6	6.7 ± 0.4

Data are expressed as mean +/- standard error of the mean of 16 replicates. * represents a significant difference from control at $P<0.05$ using Students t-test. ND is not determined.

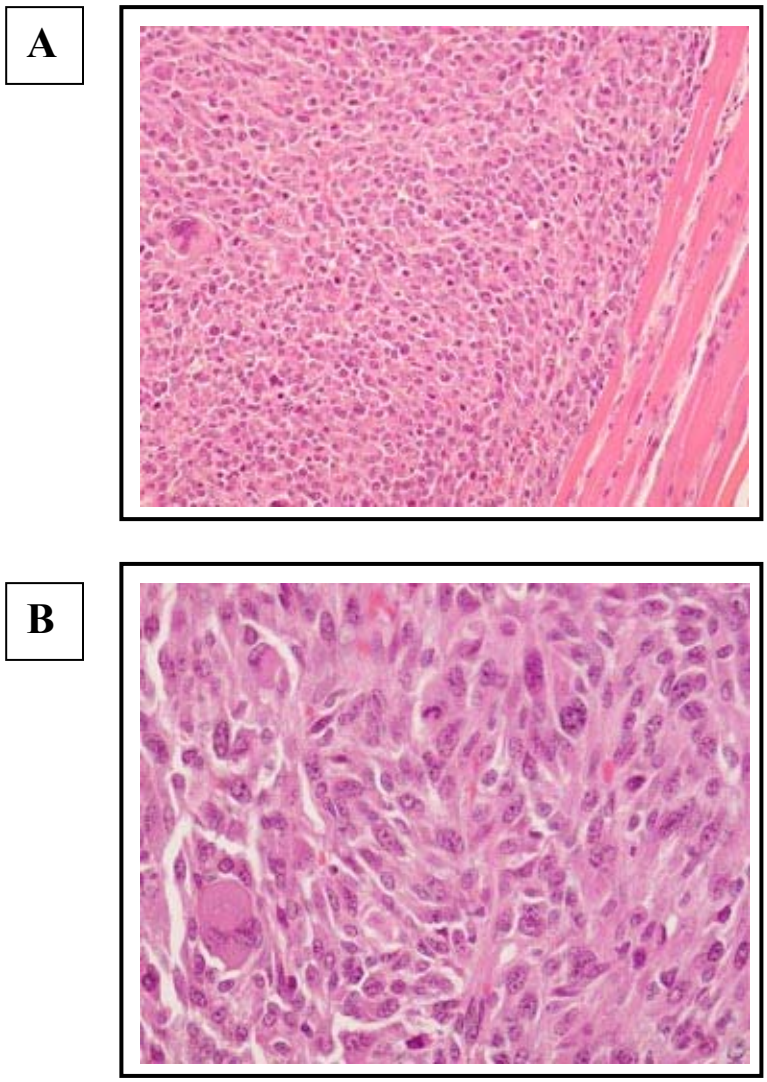


Figure 7. Tumor histopathology.
Panel A: tumor cells infiltrating muscle fibers.
Panel B: Pleomorphic nature of tumor cells.

Figure 8. Immunohistochemical Analysis of Muscle Tumor Section. Photomicrograph shows staining of tumor cells with desmin antibody indicating that tumor is derived from muscle cells.

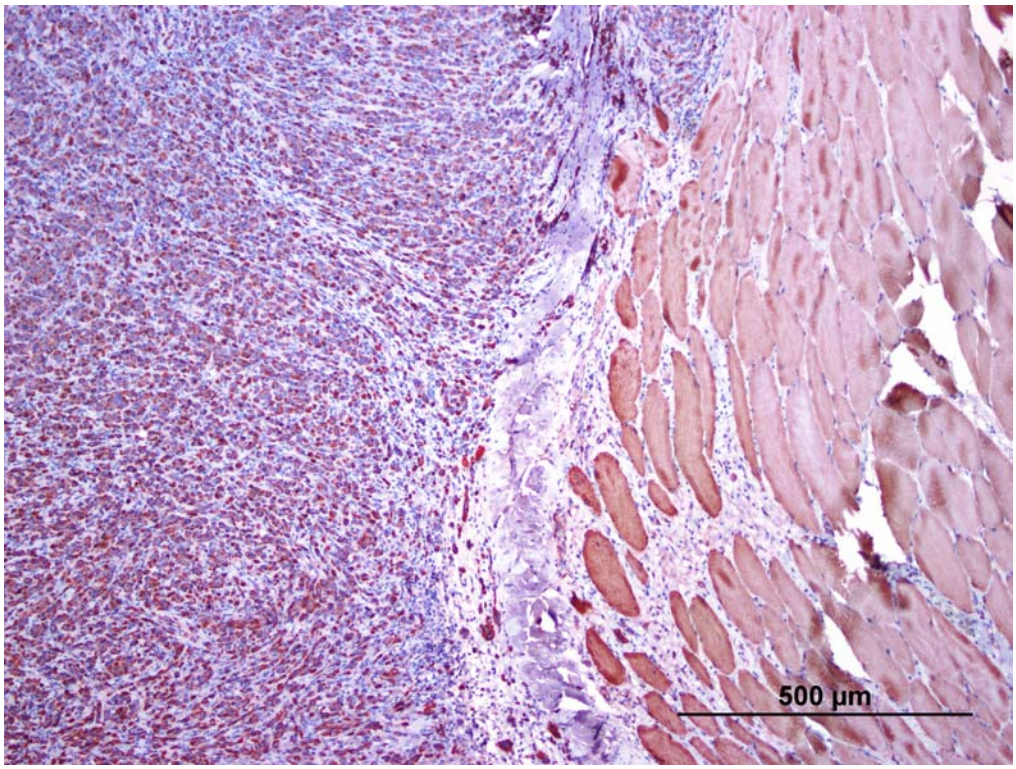


Figure 9. Masson Trichrome Stain of WA-Induced Tumor

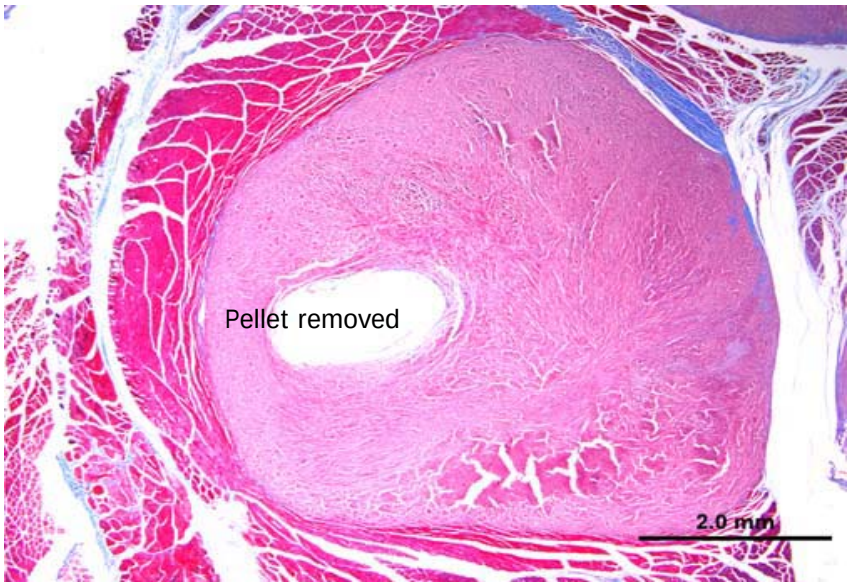


Figure 10. Sarcoma Infiltration into Skeletal Muscle.

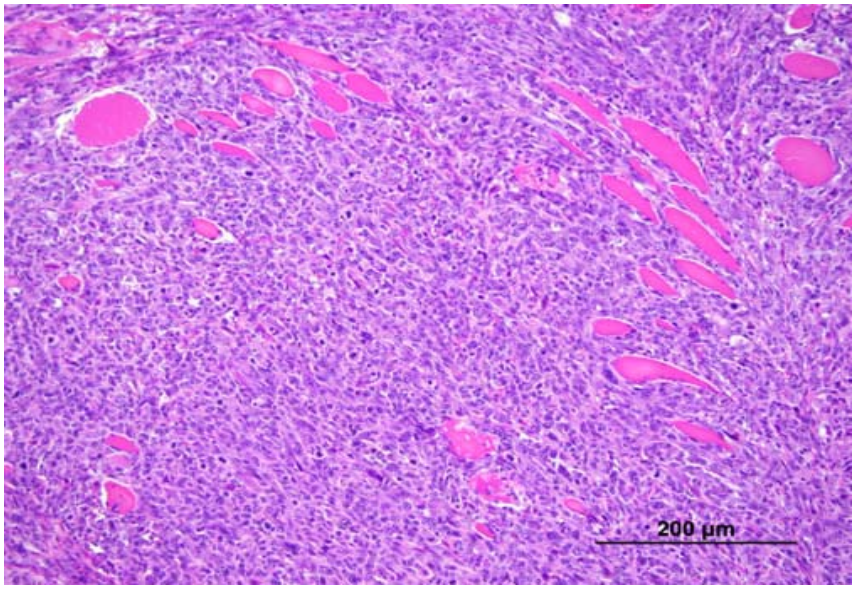


Figure 11. Sarcoma-Induced Muscle Degeneration

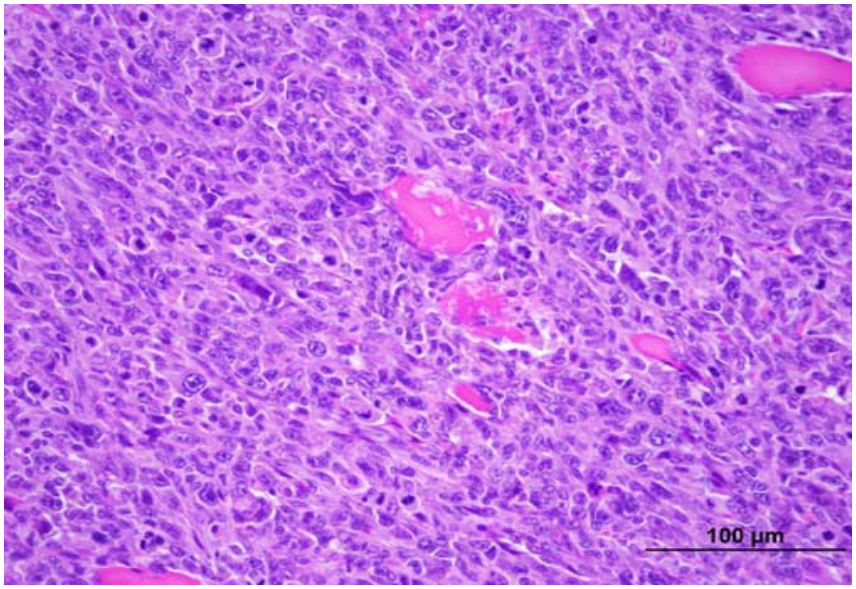


Figure 12. Variable Pattern of WA-Induced Rhabdomyosarcoma Showing Classic or Pleomorphic Rhabdomyosarcoma (lower left corner) and Embryonal Rhabdomyosarcoma (upper right corner)

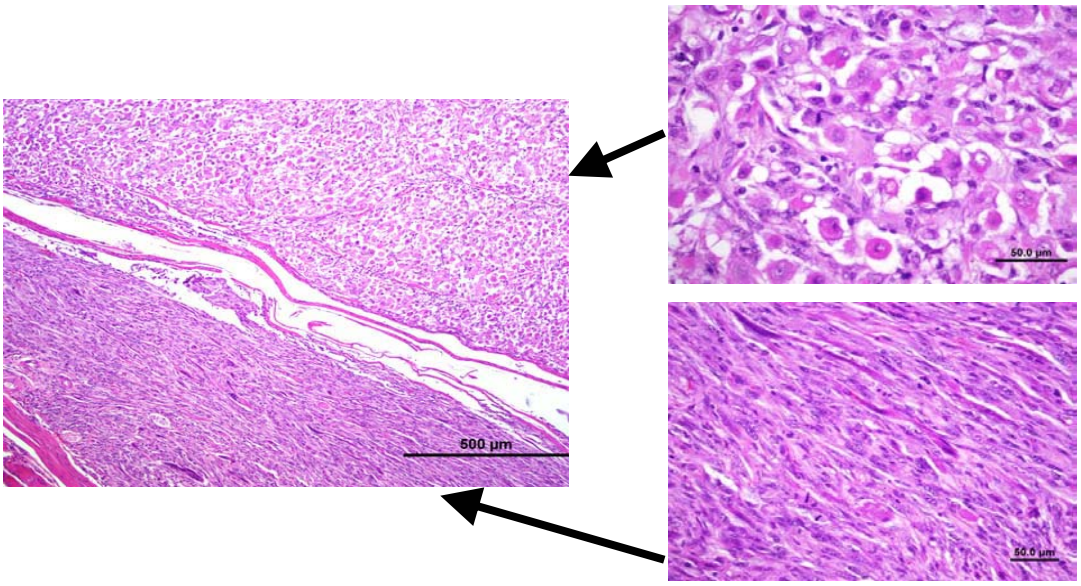


Figure 13. Neoplastic Spindle Cells and Giant Neoplastic Cells

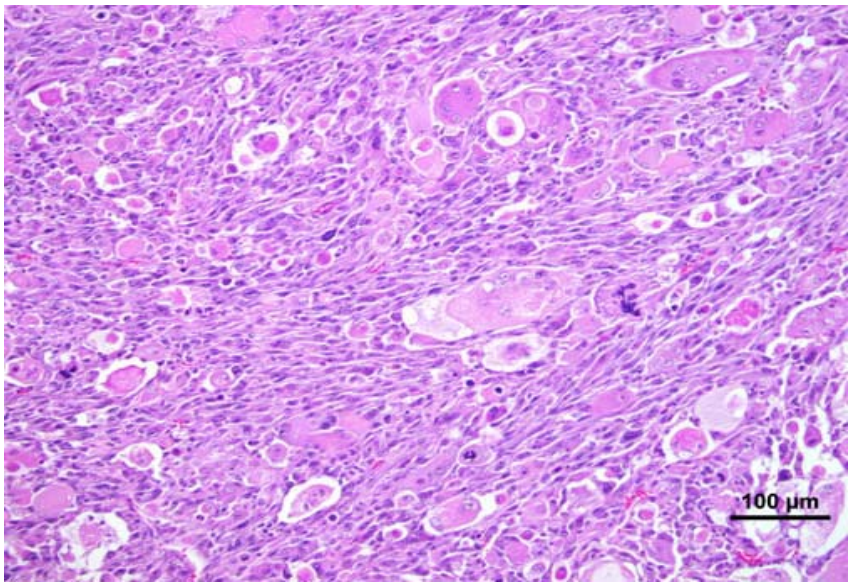


Figure 14. Pleomorphic Cells

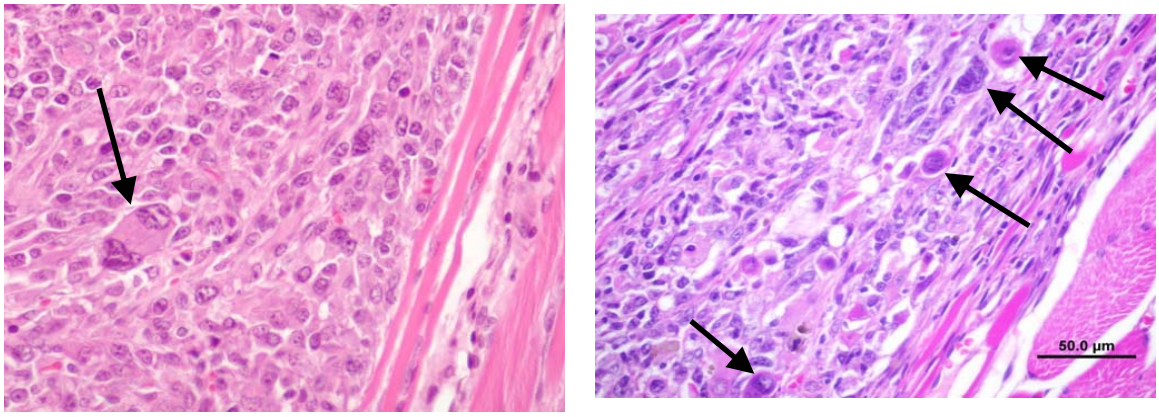


Figure 15. Vacuolated Spider Cells

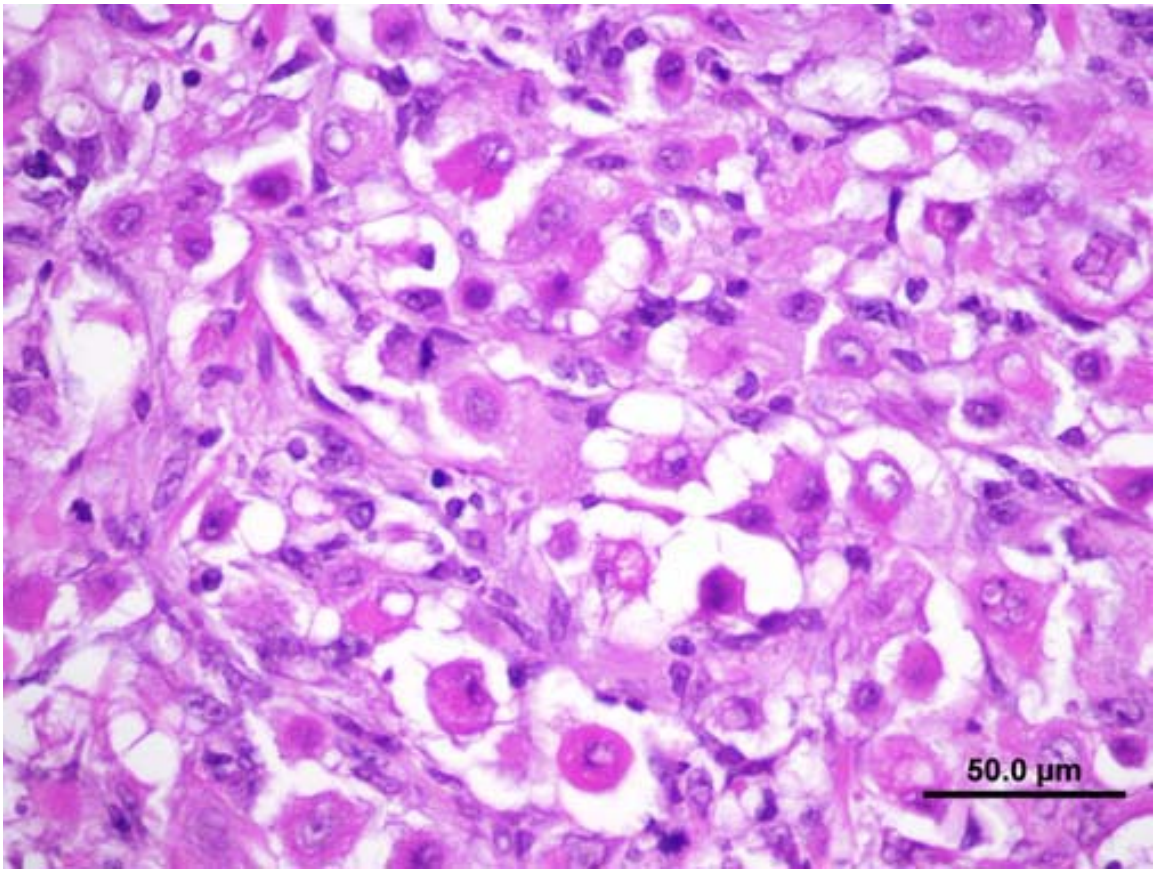


Figure 16. Multinucleate Giant Cells

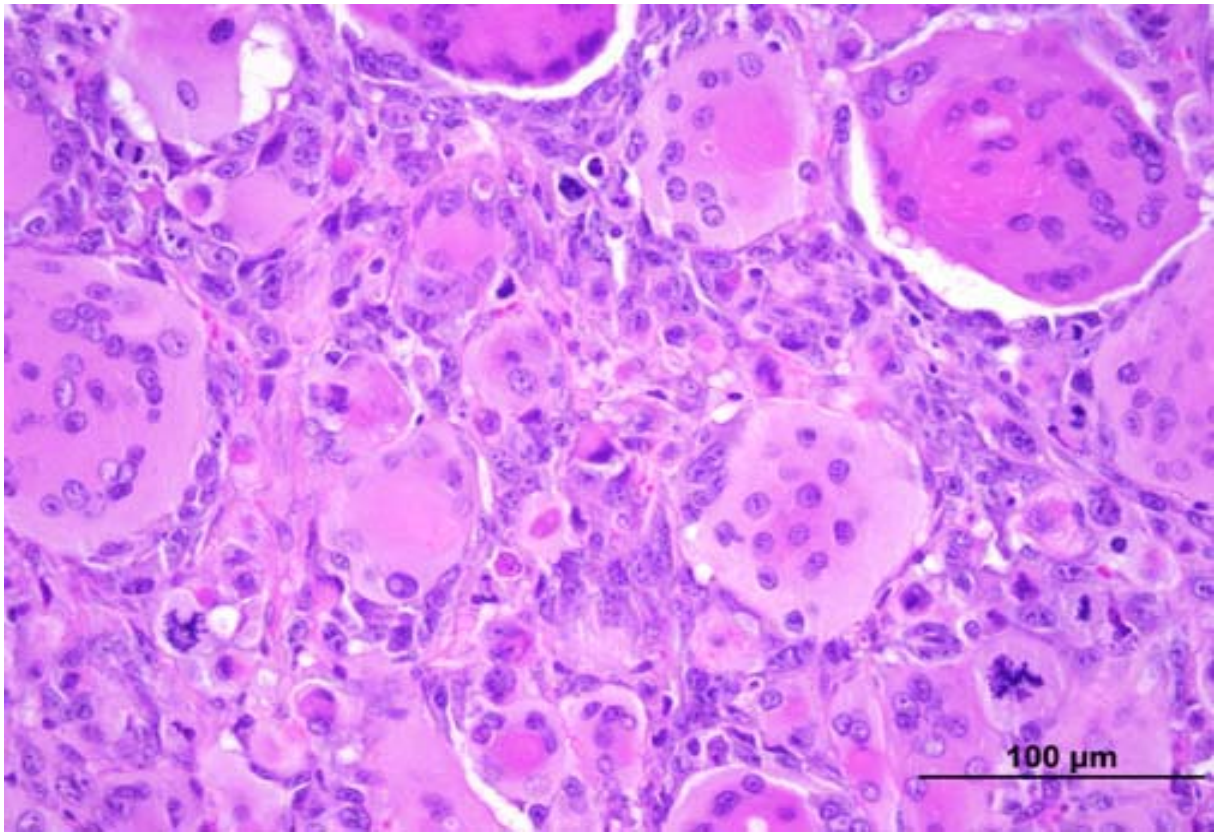


Figure 17. Multinucleate Giant and Strap Cells.

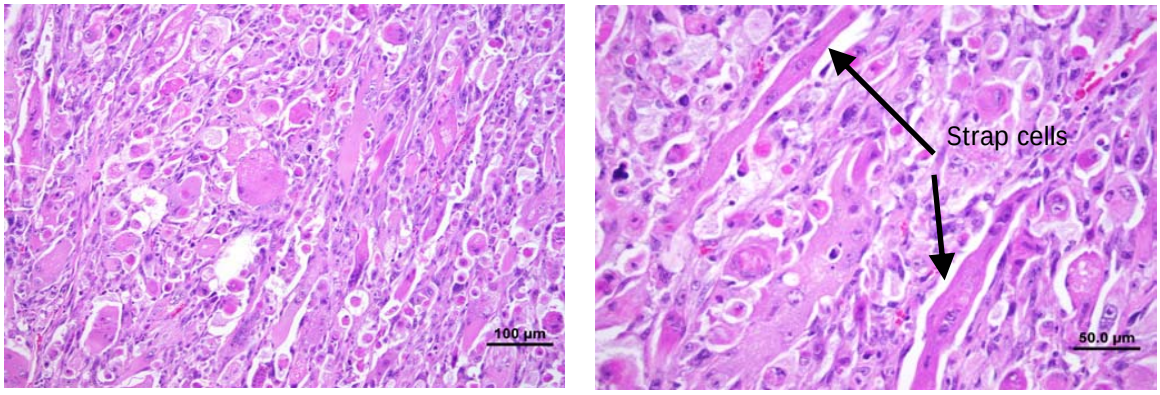


Figure 18. Hematoxylin and Eosin-Stained Section of Pellet Implantation Site from Leg of Rat from 3 Month Low-Dose WA Group. The upper hole was the site of a WA pellet, while the lower hole (with the size bar) contained a tantalum pellet. Note the initial development of neoplastic cells surrounding the WA pellet.

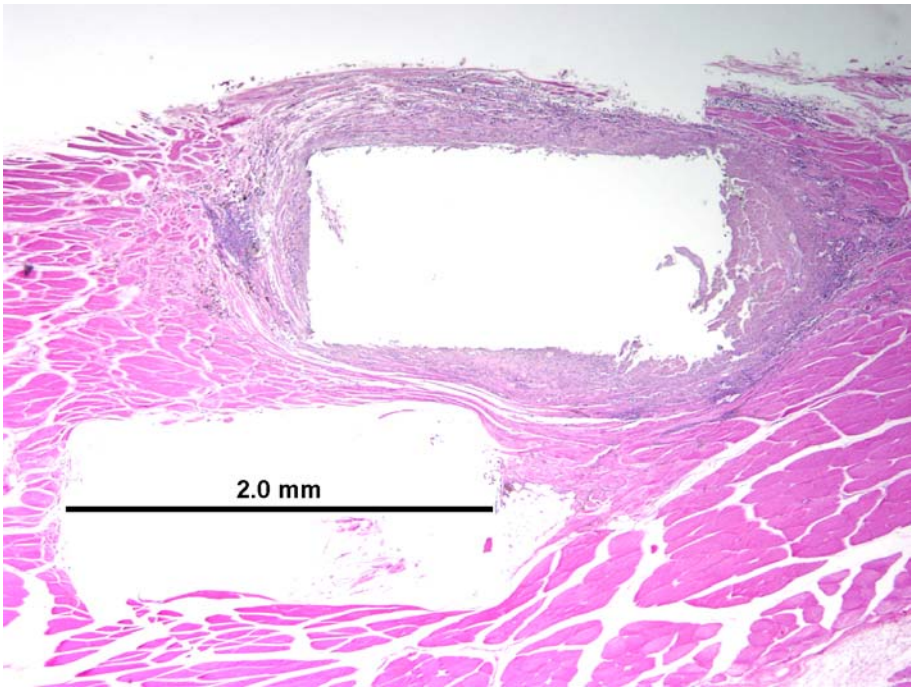


Figure 19. Splenic Red Pulp

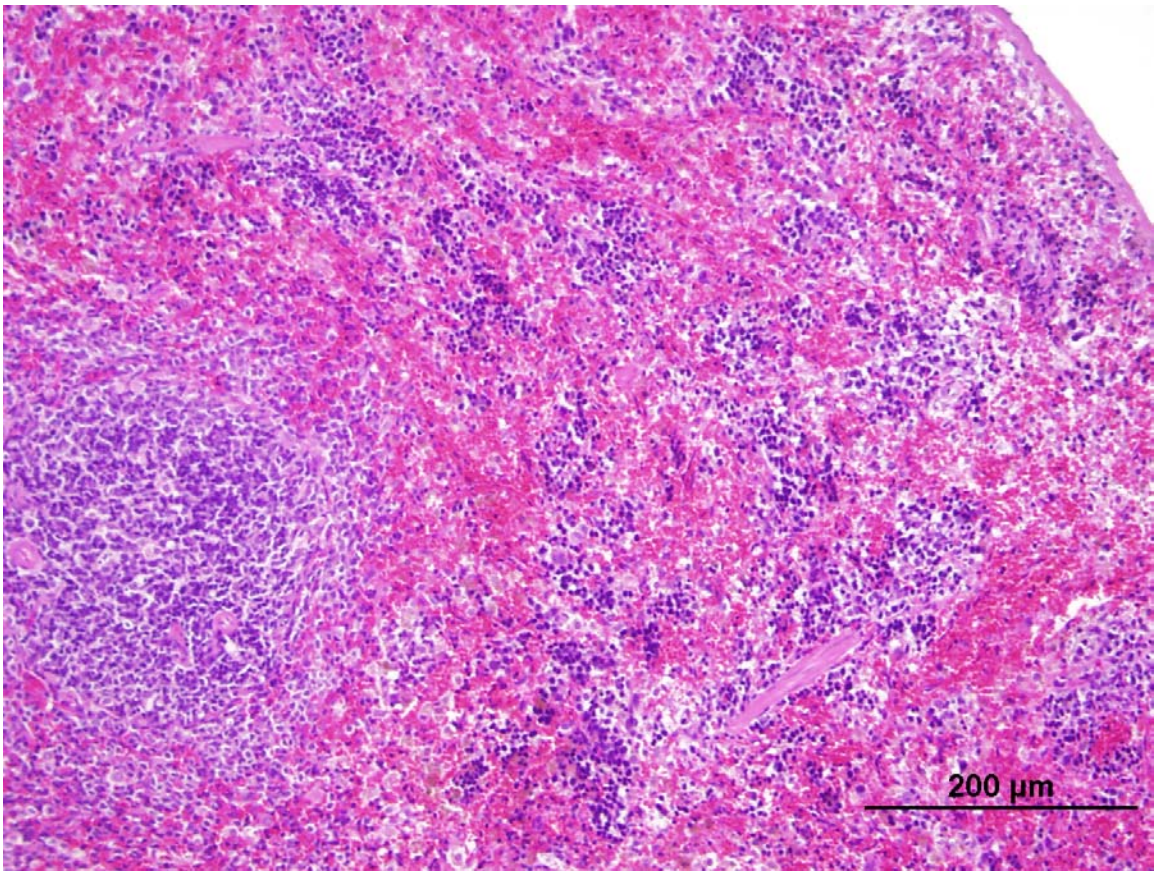


Figure 20. Interstitial cell tumor (testes)

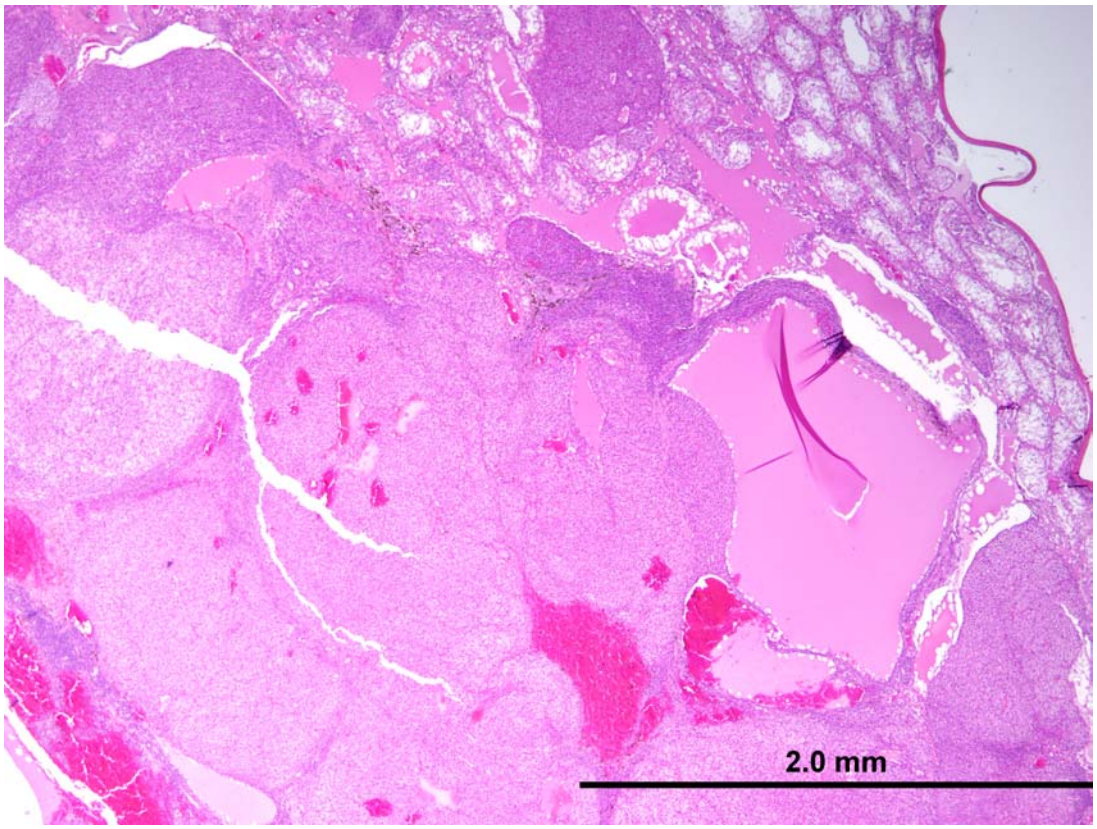


Figure 21. Chronic nodular granulomatous steatitis

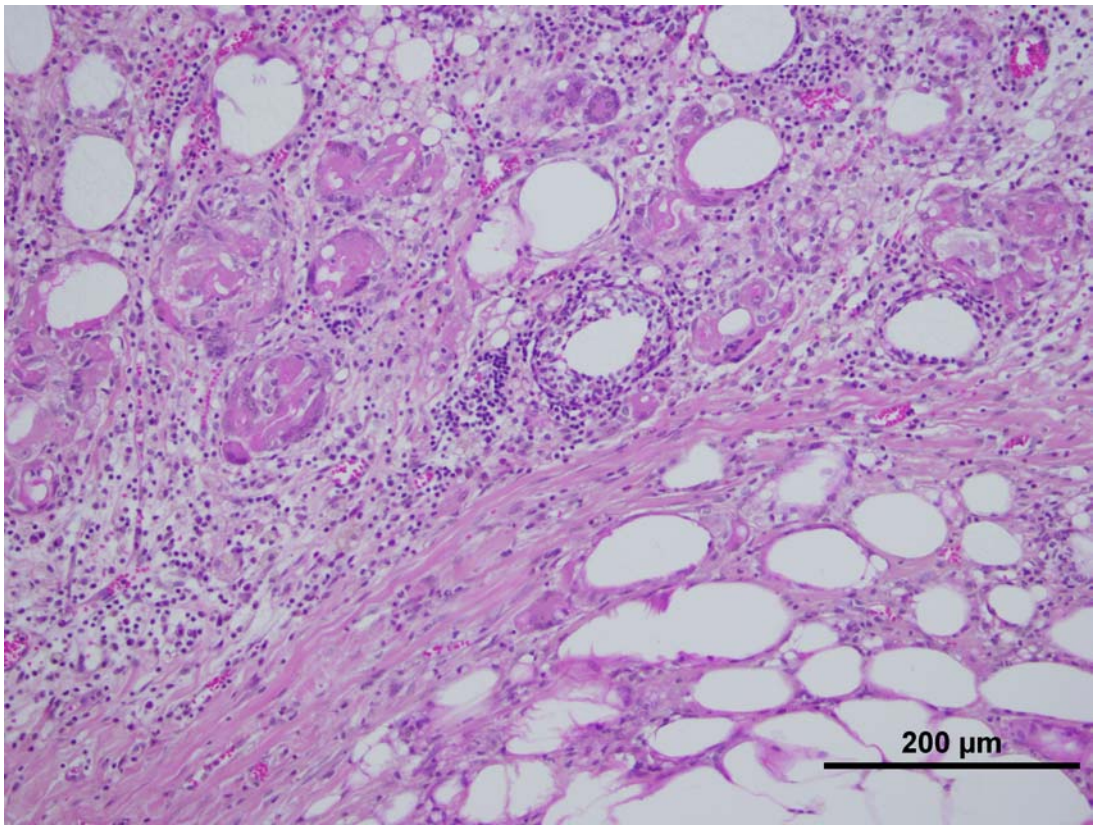


Figure 22. Malignant pheochromocytoma (24 month DU high-dose rat)

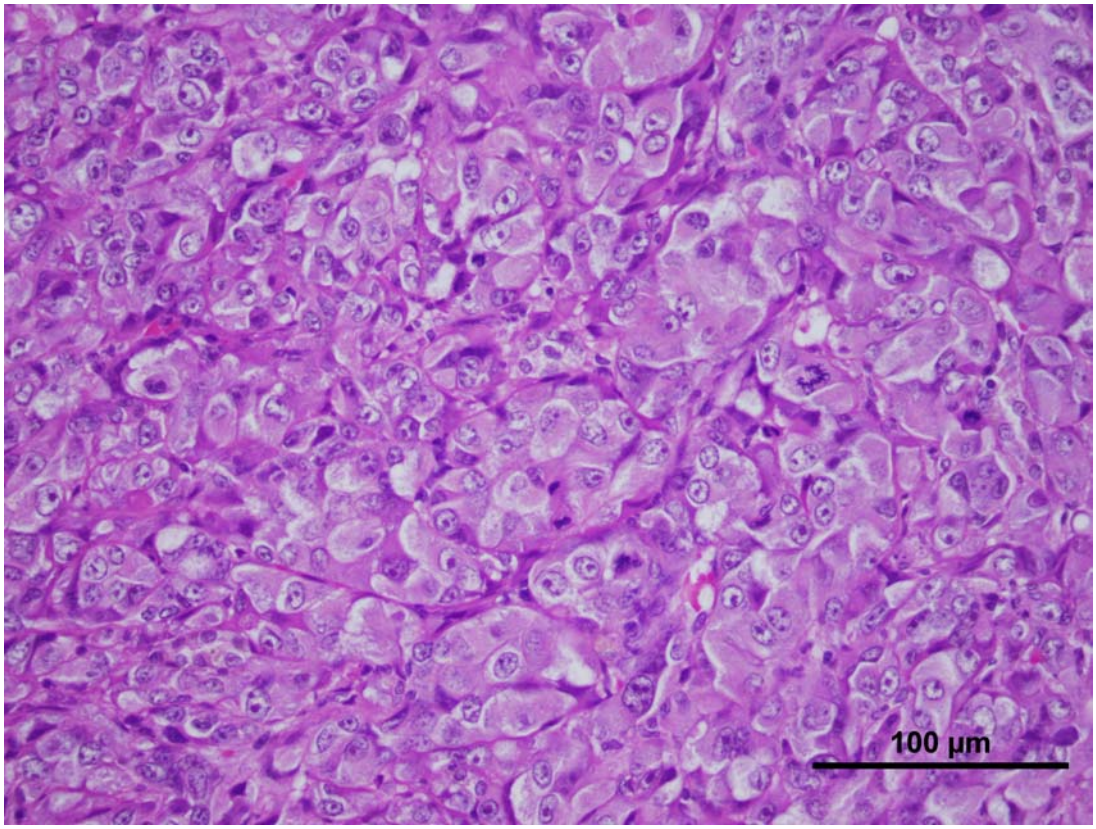


Figure 23. Renal tubular carcinoma (24 month DU high-dose rat)

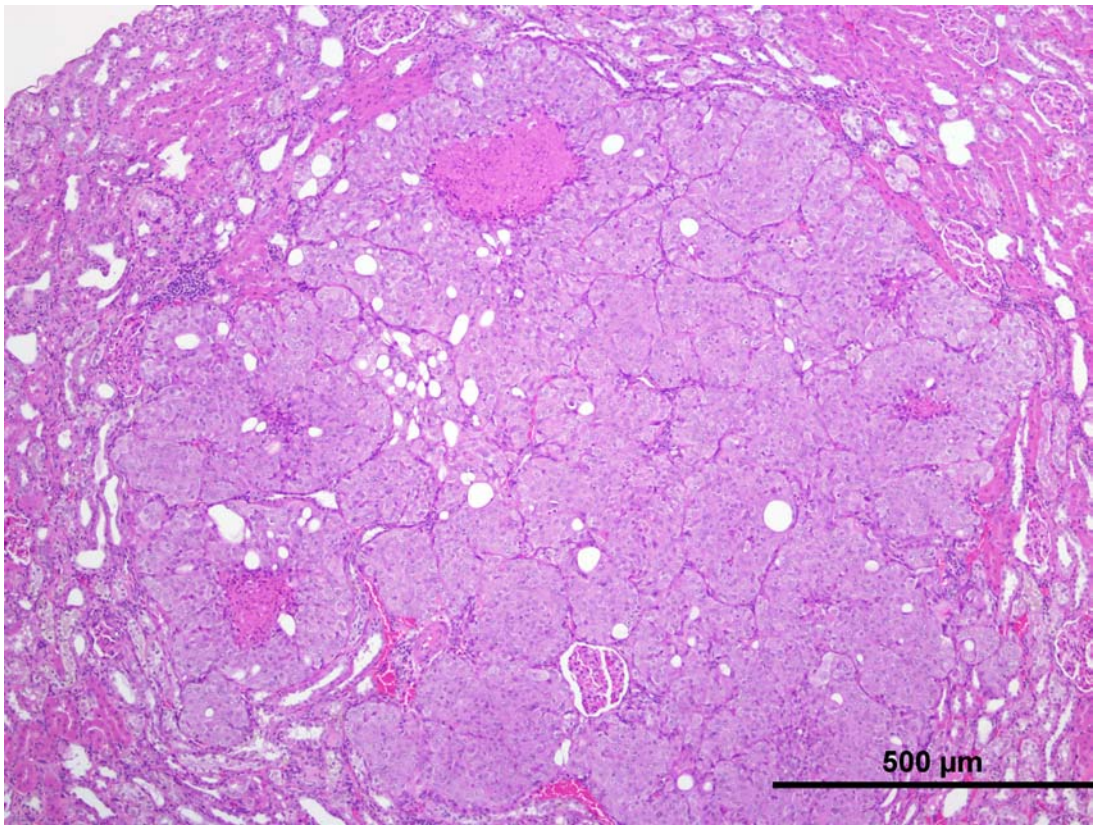


Figure 24. Renal mesenchymal tumor (24 month DU high-dose rat)

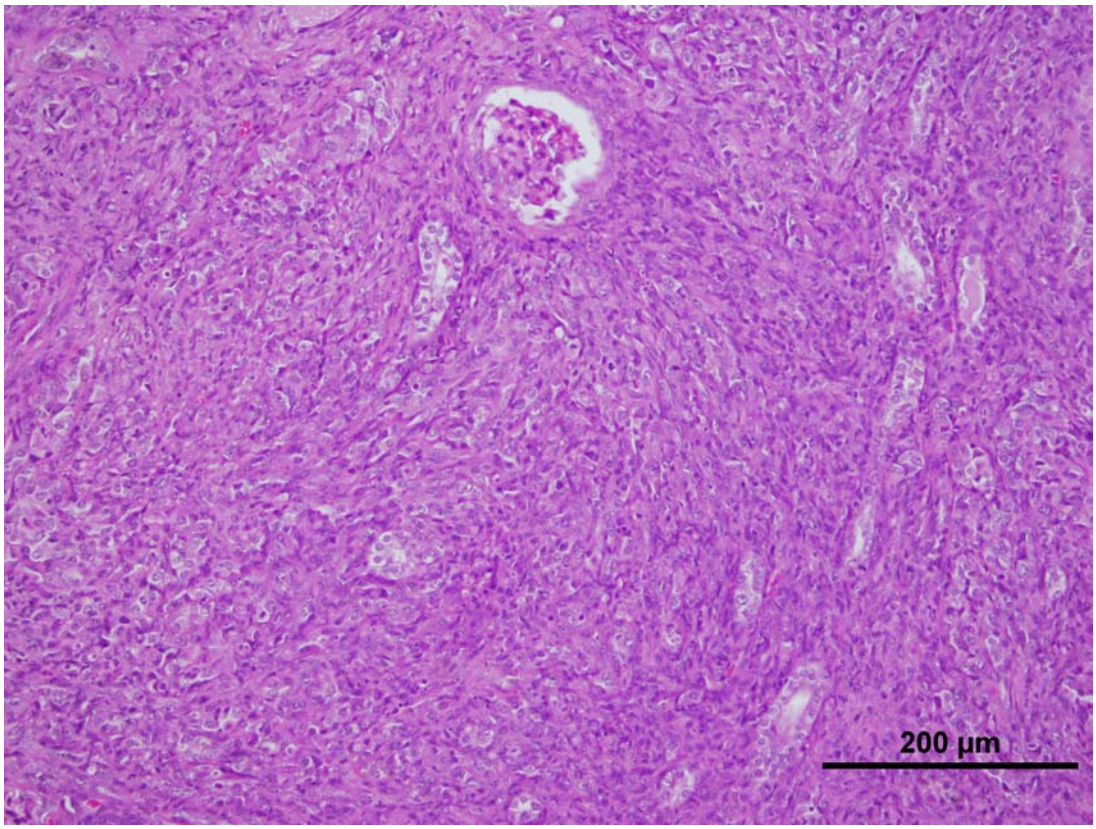
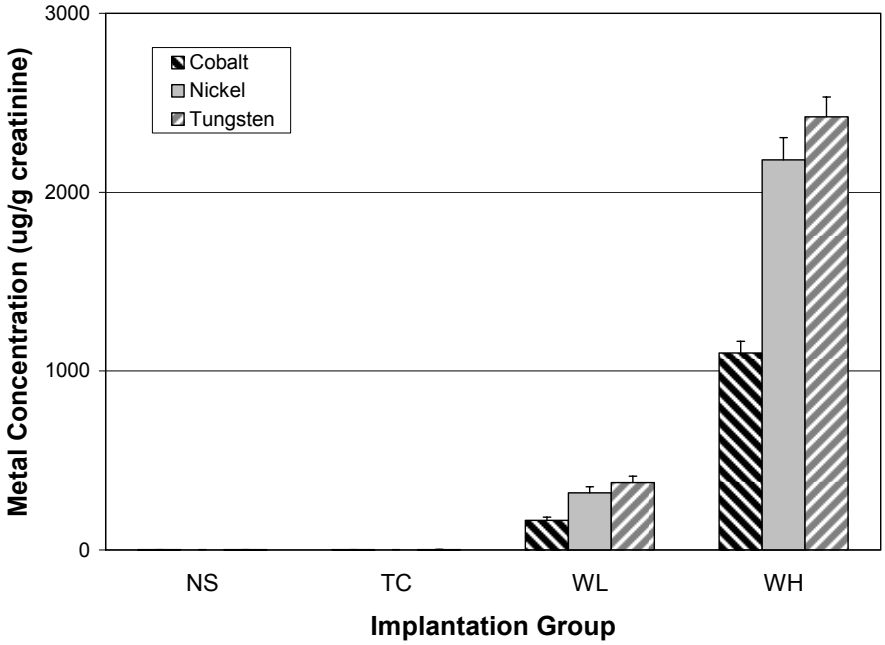


Table 27. ThermoElectron PQ ExCell ICP-MS Operating Conditions and Parameters

Instrument	
Nebulizer type	Concentric
Spray chamber	Conical, with impact bead
Sampler cone	Platinum, 1 mm orifice diameter
Skimmer cone	Platinum, 0.7 mm orifice diameter
Sample uptake rate	1.0 ml/min
Sample read delay	60 sec
Plasma conditions	
Forward power	1350 W
Plasma argon gas flow	13 L/min
Auxiliary argon gas flow	0.9 L/min
Nebulizer gas flow	1.0 L/min
Mass spectrometer settings	
Scanning mode	Main run (peak jump mode)
Sweeps	100
Channels/mass	1
Acquisition time	18 sec
Number of readings/replicate	5
Number of replicates	1

A



B

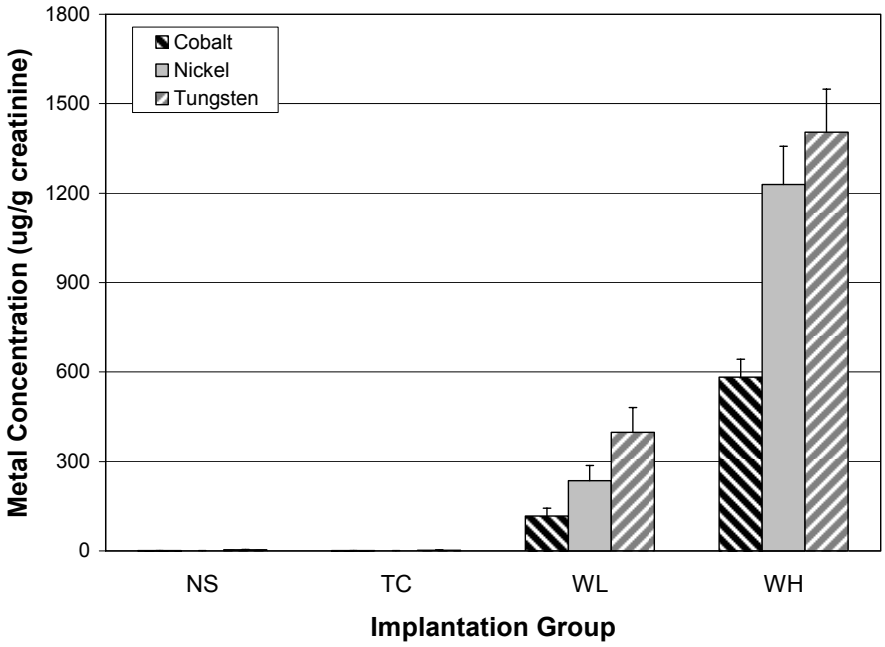


Figure 25. Urine Metal (Co, Ni, W) Concentrations: 1 Month (Panel A) and 3 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

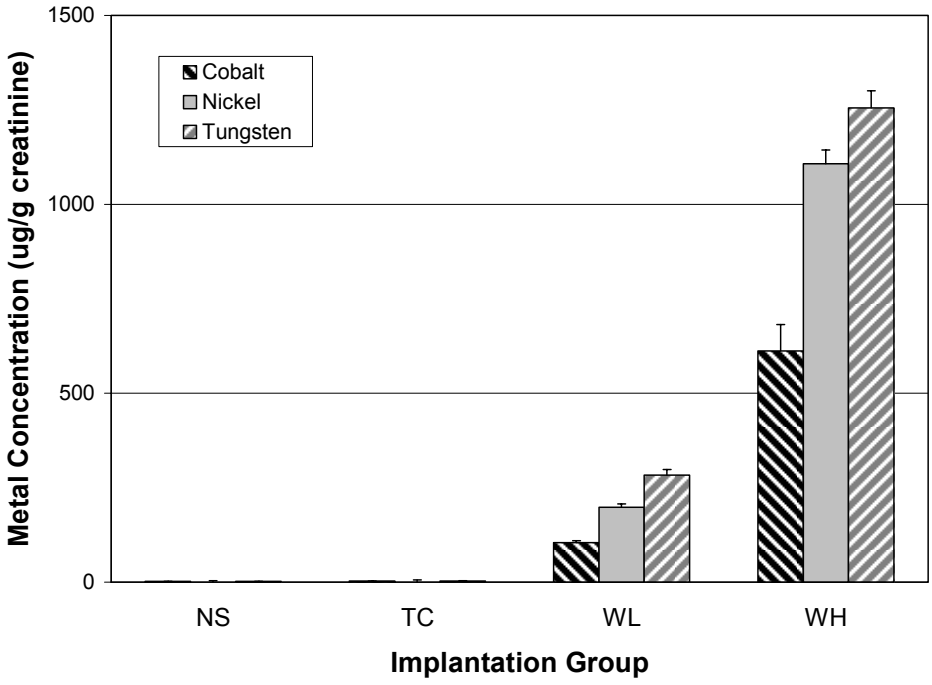
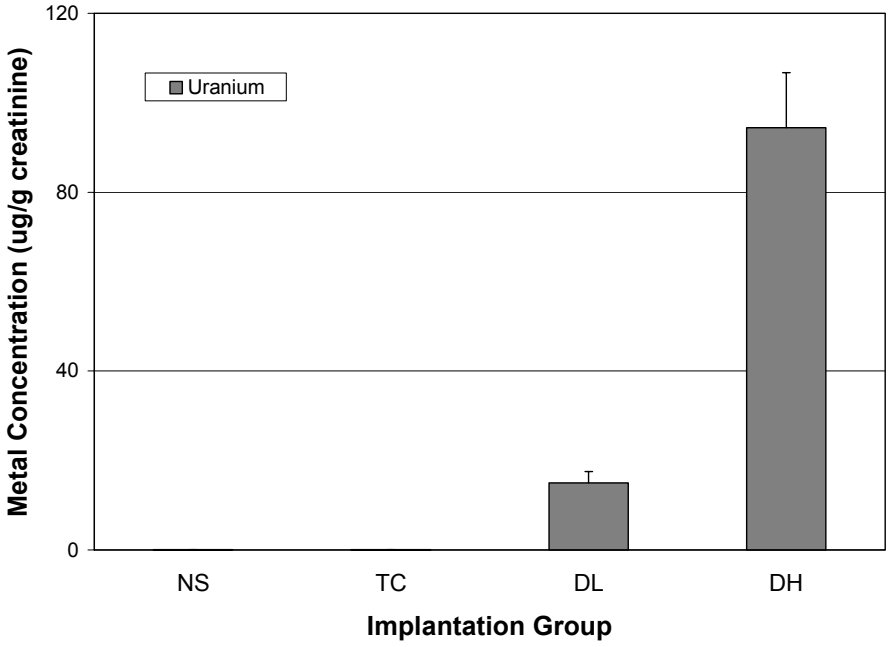


Figure 26. Urine Metal (Co, Ni, W) Concentrations: 6 Month Group. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

A



B

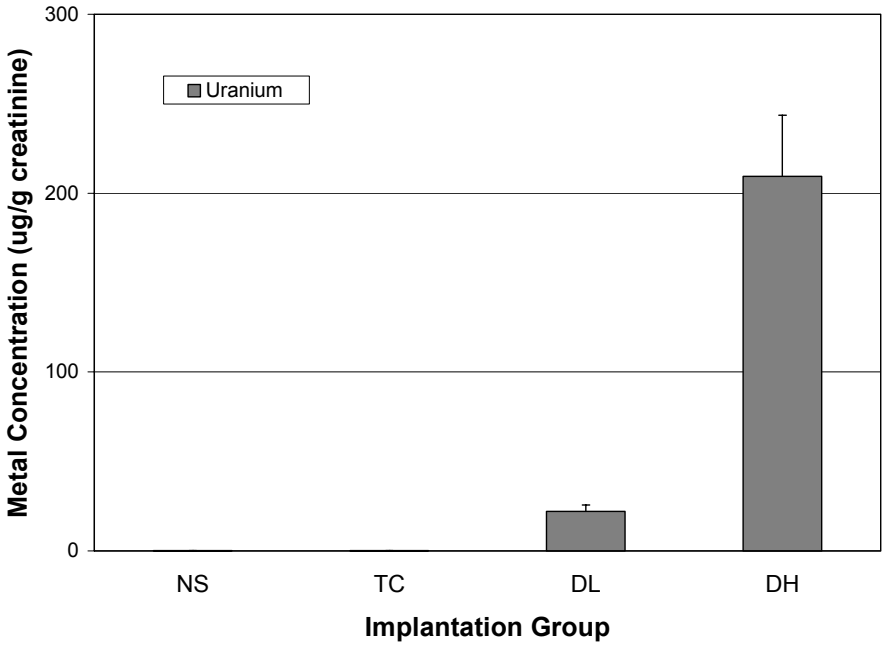
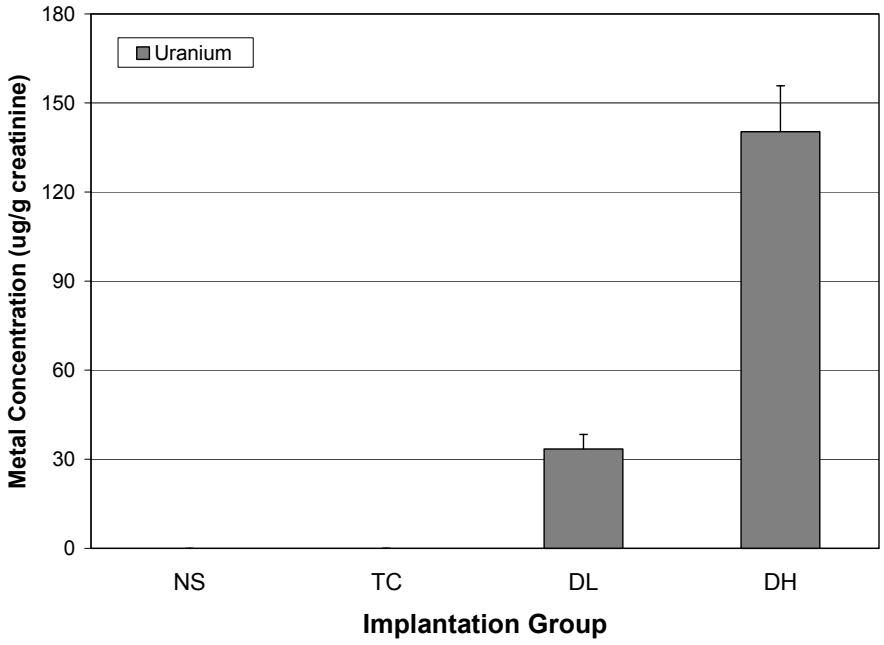


Figure 27. Urine Uranium Concentrations: 1 Month (Panel A) and 3 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

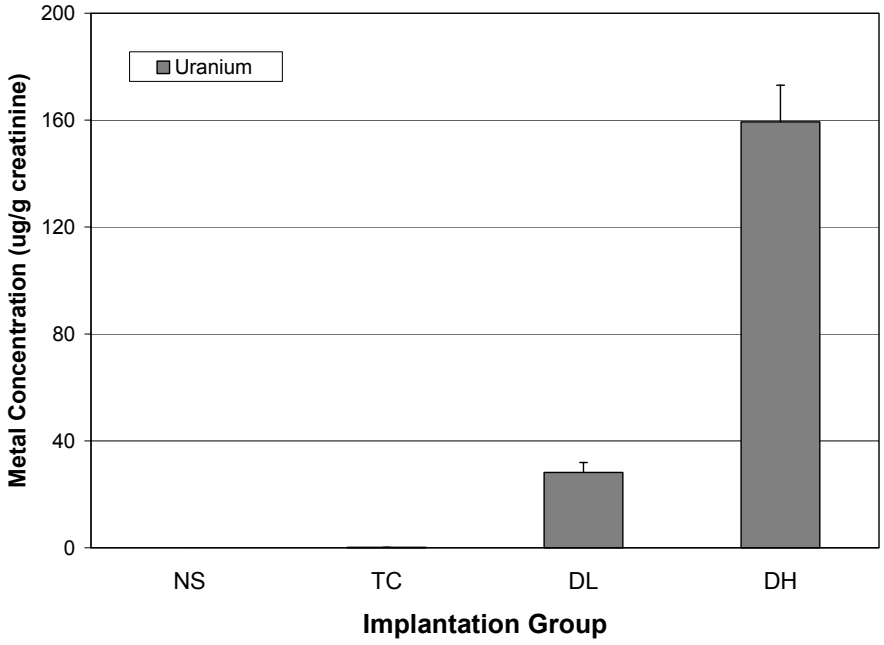


Figure 28. Urine Uranium Concentrations: 6 Month (Panel A) and 12 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

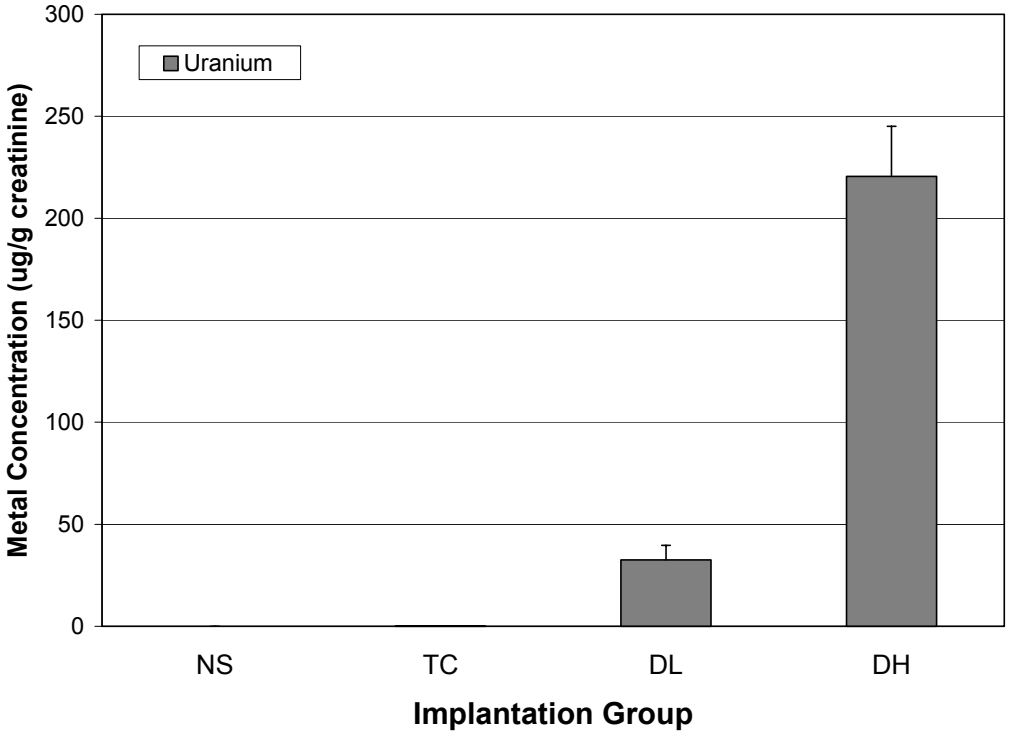
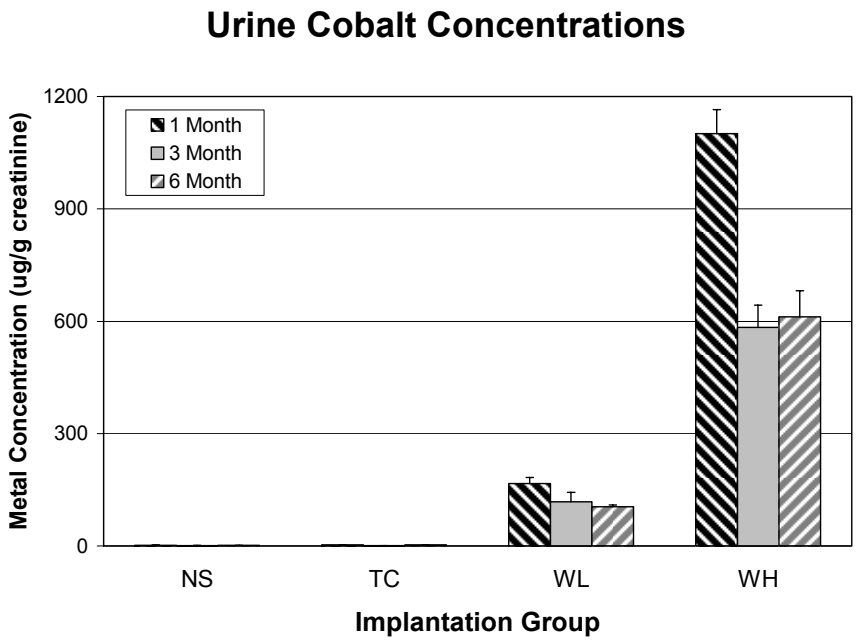


Figure 29. Urine Uranium Concentrations: 18 Month Group. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

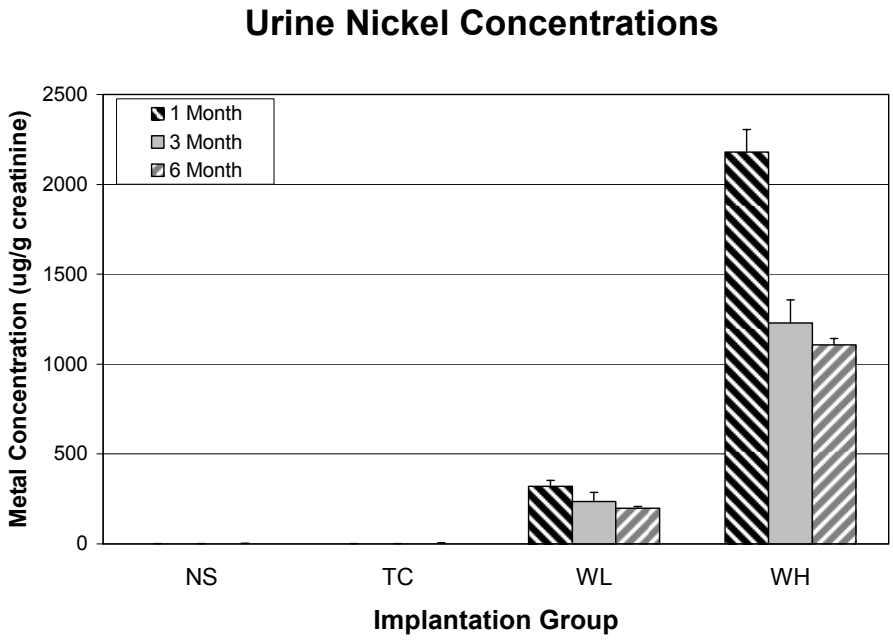
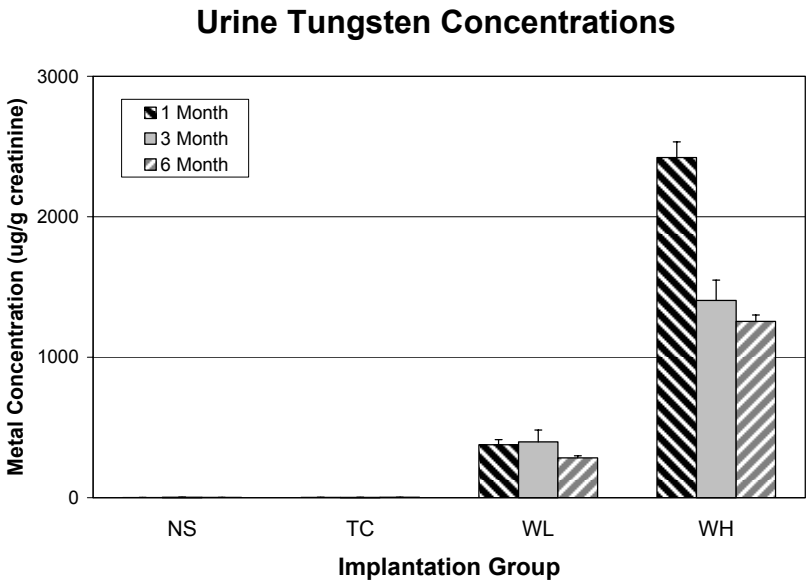


Figure 30. Urine Cobalt (Panel A) and Nickel (Panel B) Concentrations over Time. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

A



B

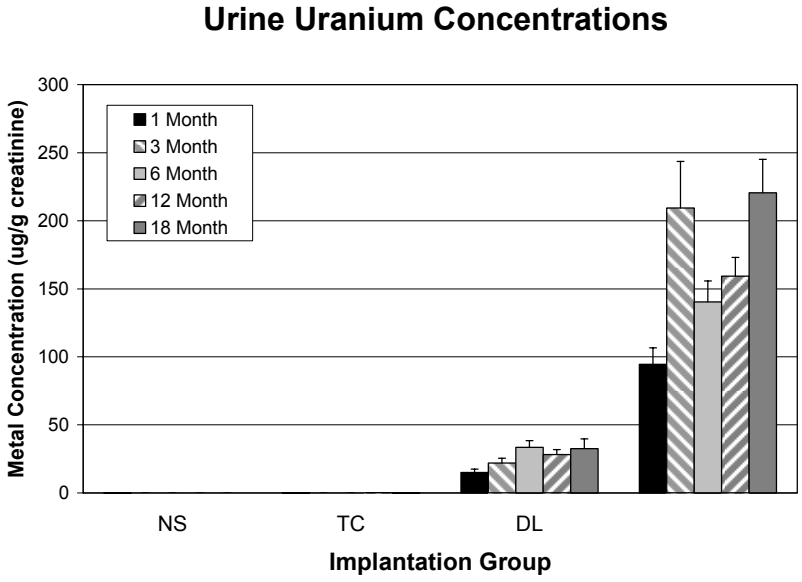
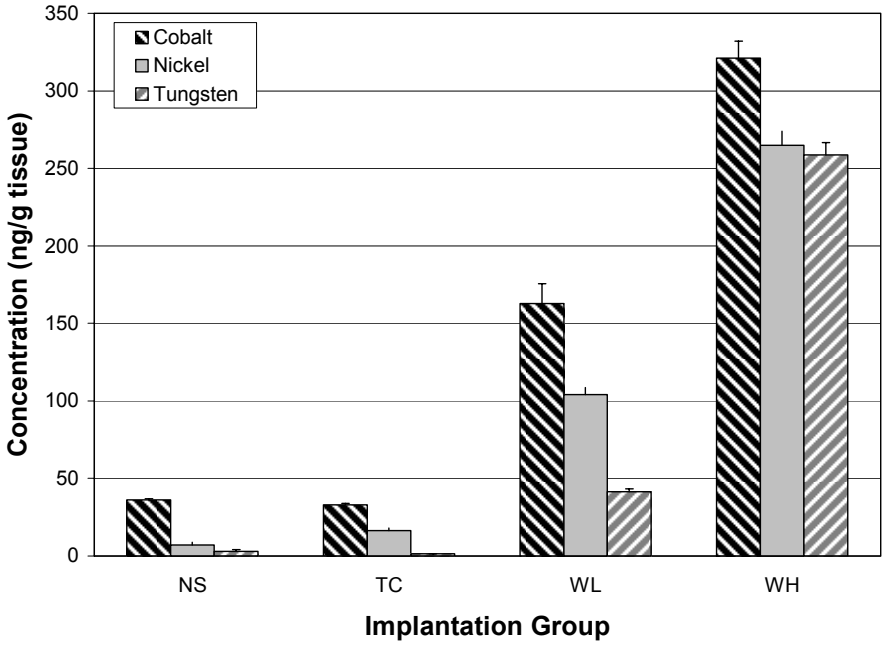


Figure 31. Urine Tungsten (Panel A) and Uranium (Panel B) Concentrations over Time. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose); DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

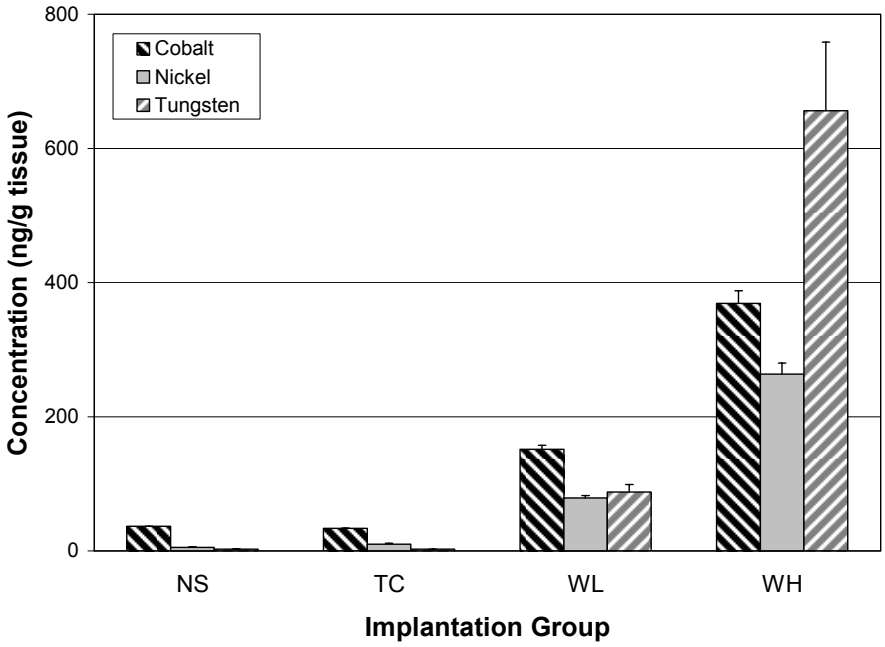


Figure 32. Kidney Metal (Co, Ni, W) Concentrations: 1 Month (Panel A) and 3 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

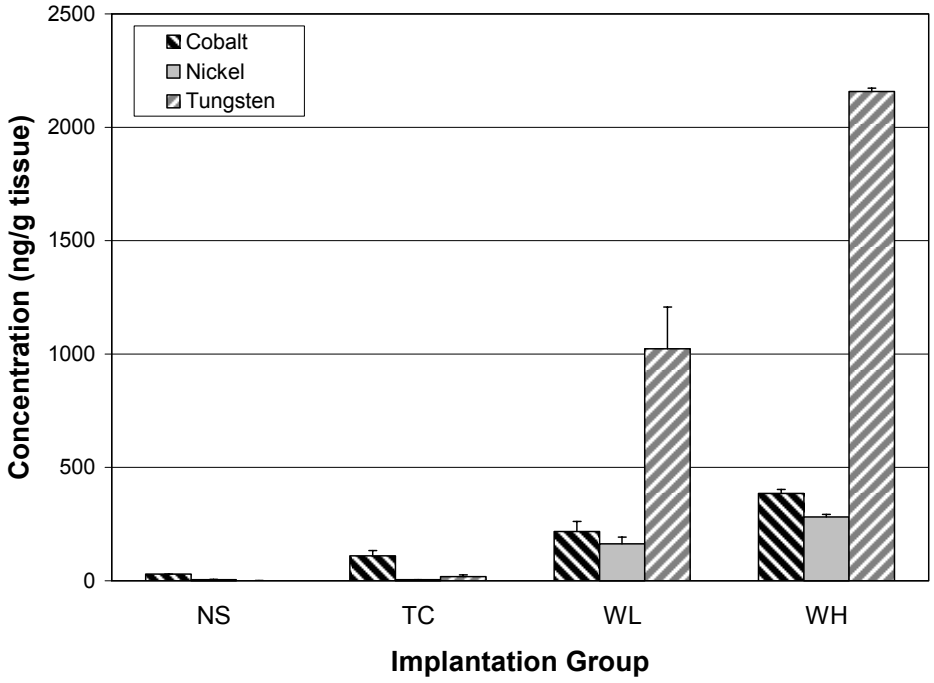
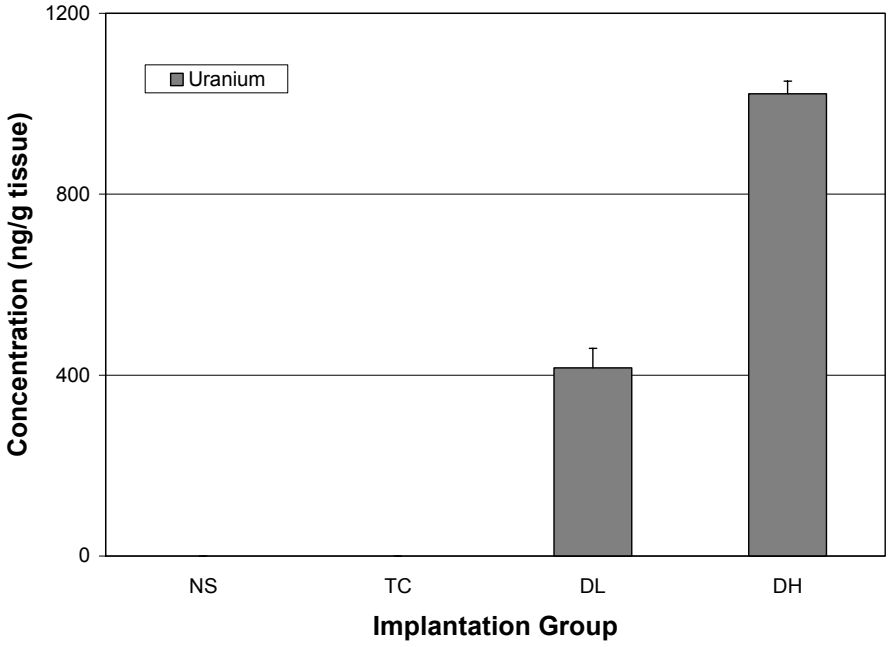


Figure 33. Kidney Metal (Co, Ni, W) Concentrations: 6 Month Group. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

A



B

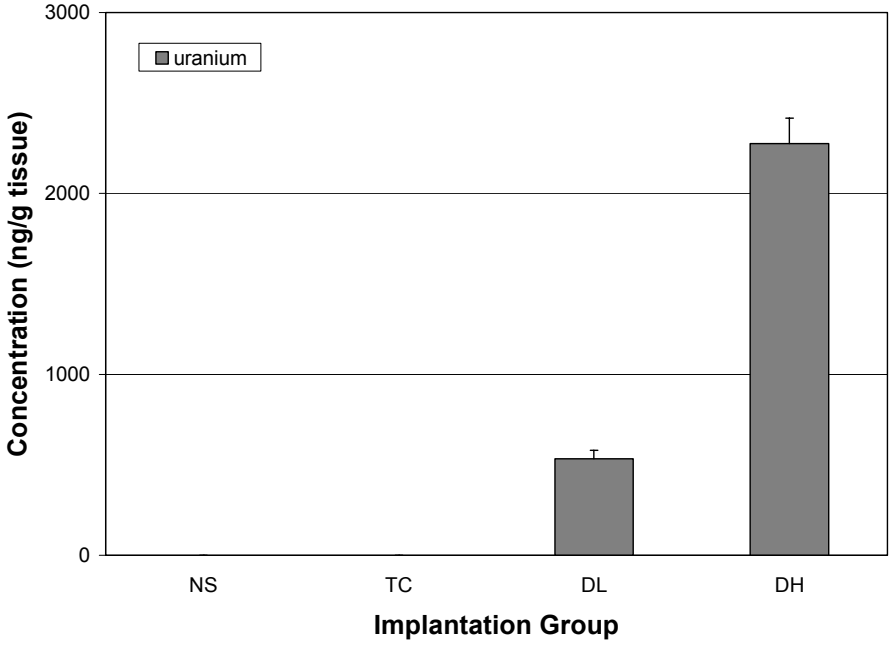
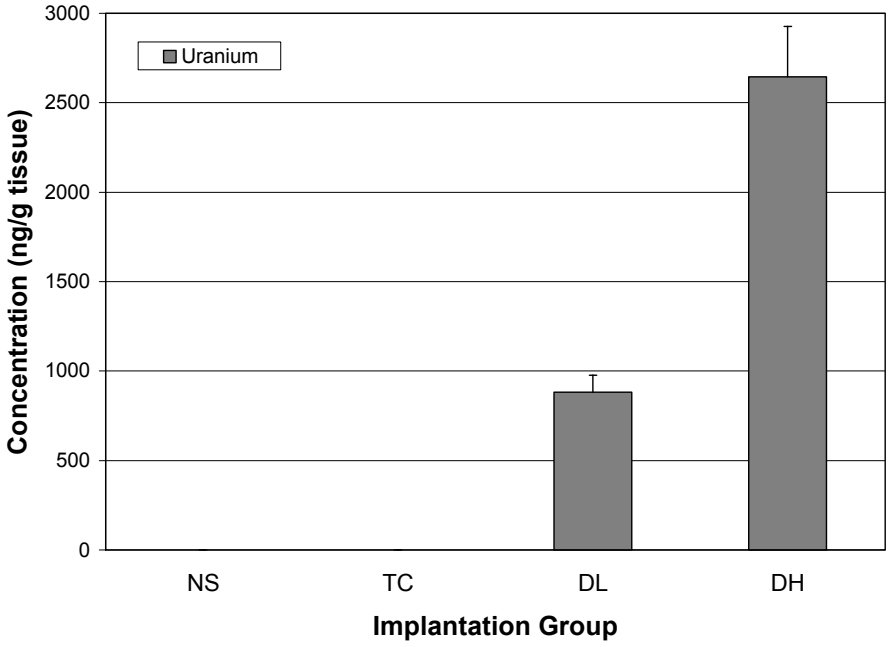


Figure 34. Kidney Uranium Concentrations: 1 Month (Panel A) and 3 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

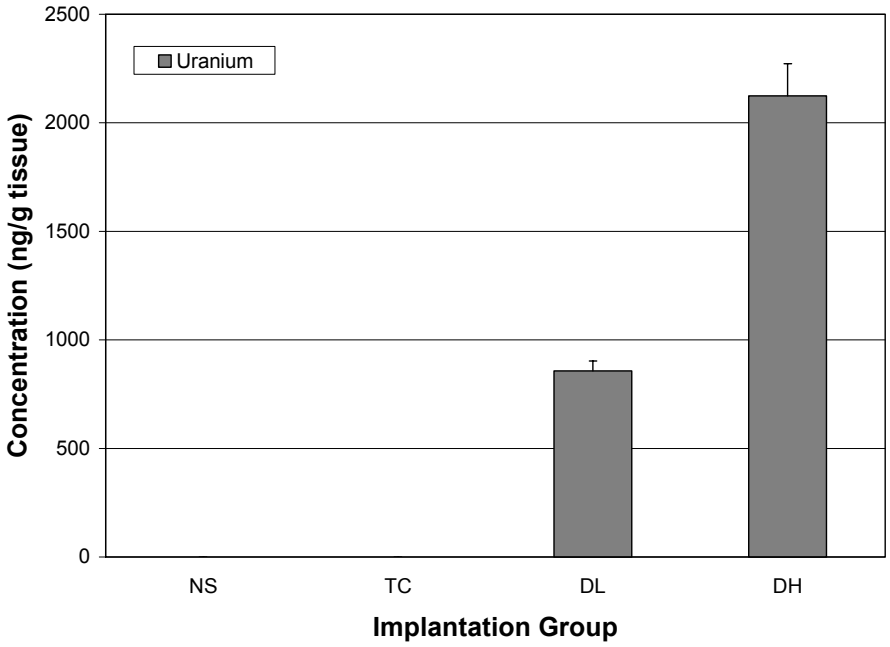


Figure 35. Kidney Uranium Concentrations: 6 Month (Panel A) and 12 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

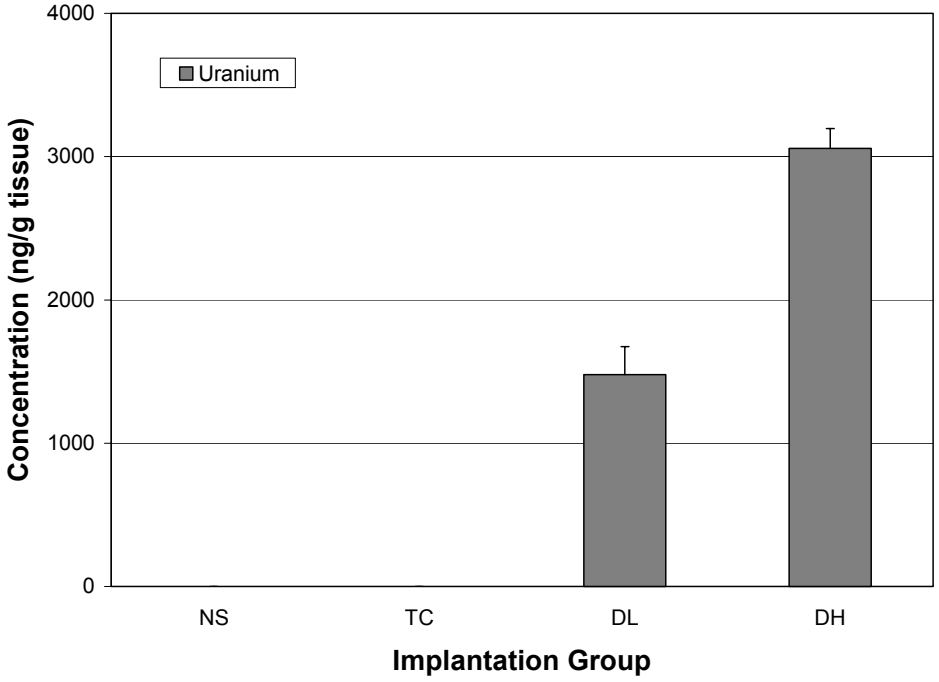
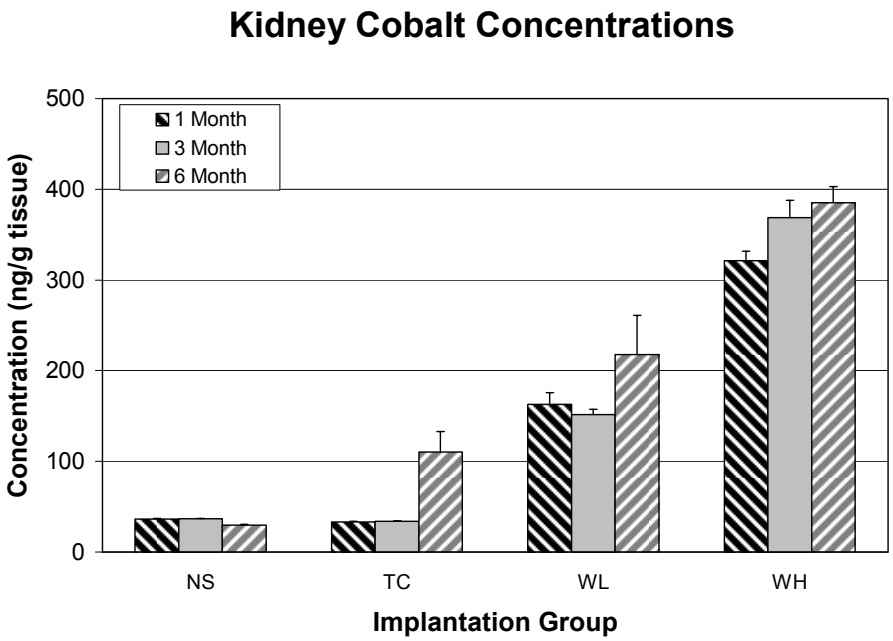


Figure 36. Kidney Uranium Concentrations: 18 Month Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

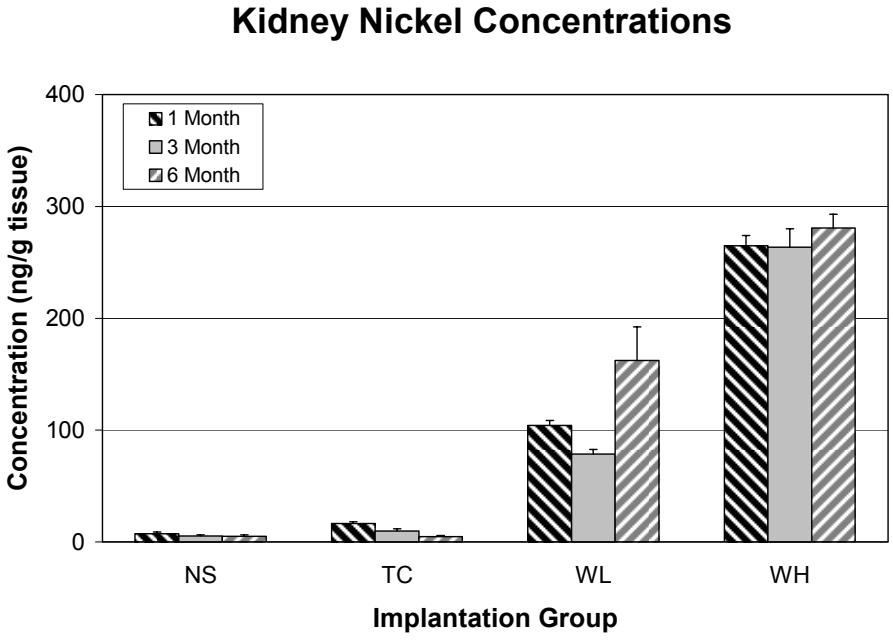
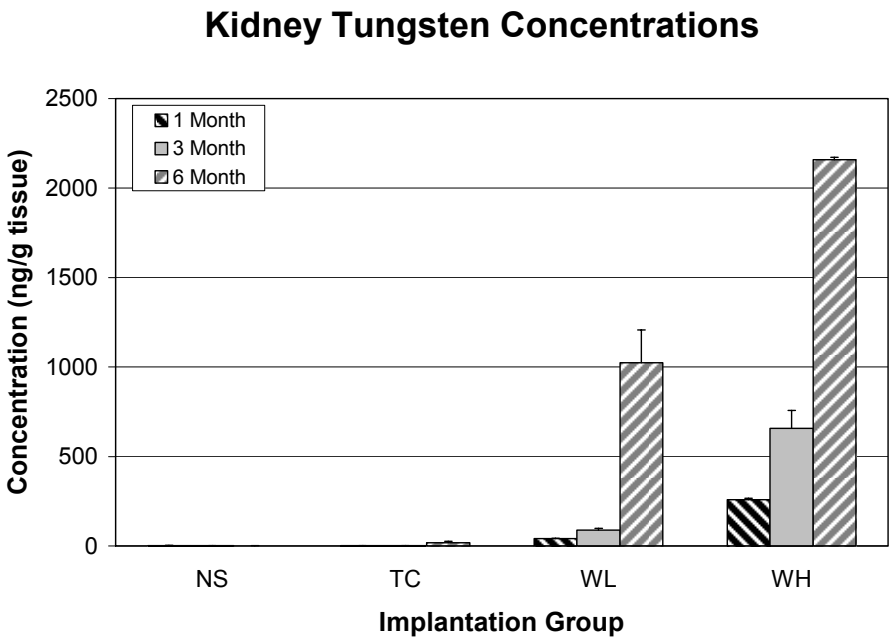


Figure 37. Kidney Cobalt (Panel A) and Nickel (Panel B) Concentrations over Time. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

A



B

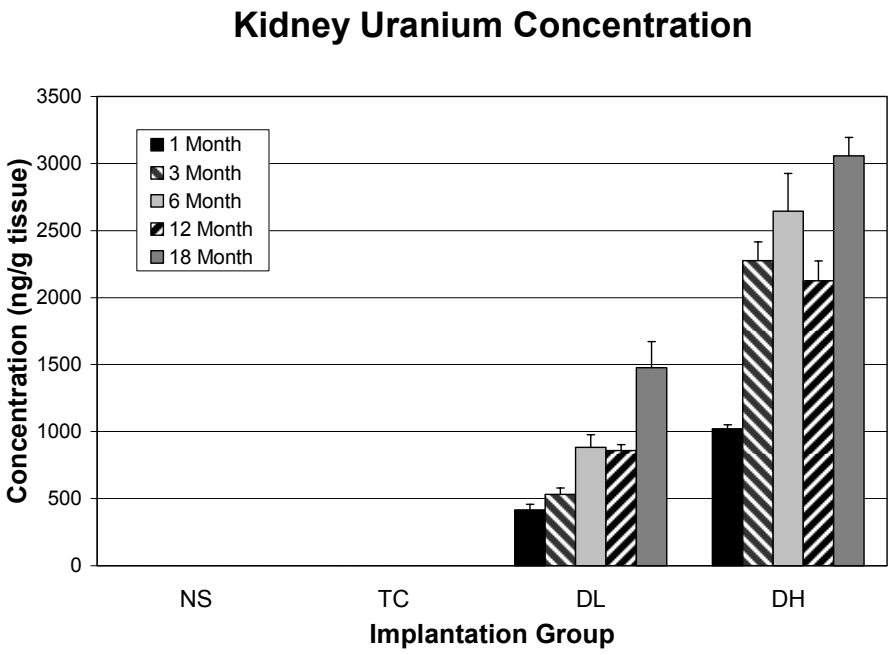
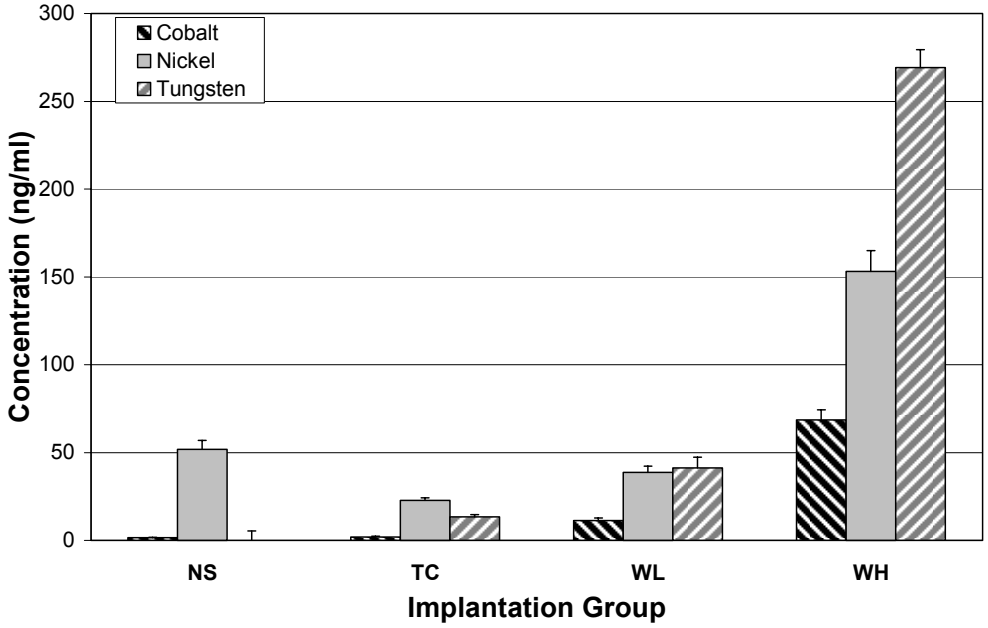


Figure 38. Kidney Tungsten (Panel A) and Uranium (Panel B) Concentrations over Time. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose); DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

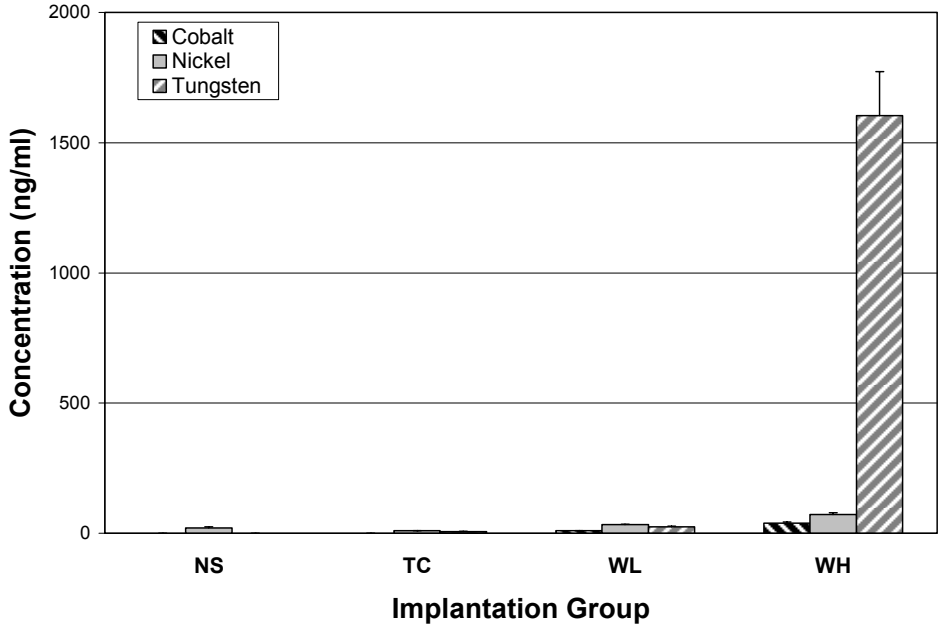


Figure 39. Serum Metal (Co, Ni, W) Concentrations: 1 Month (Panel A) and 3 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

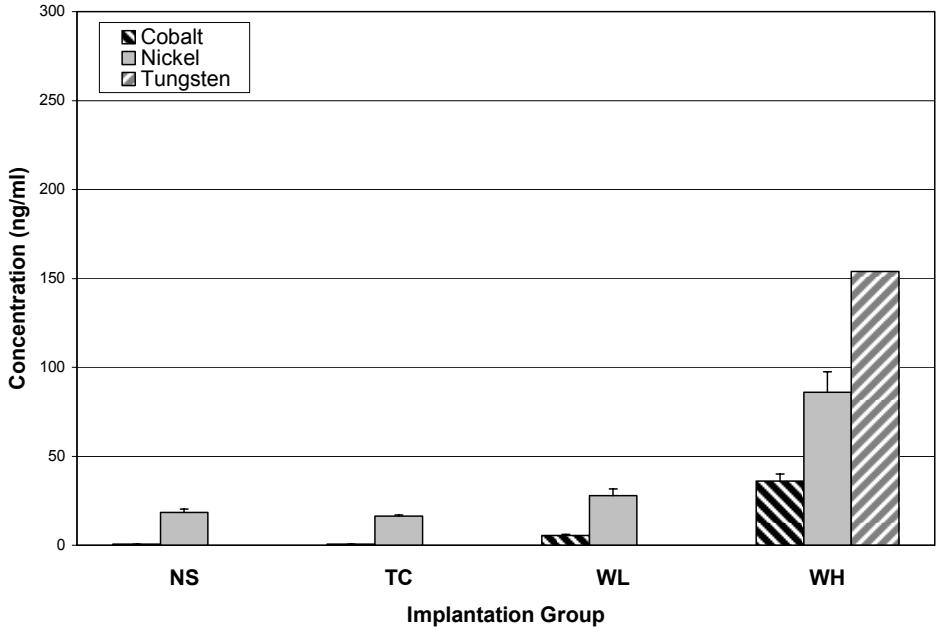
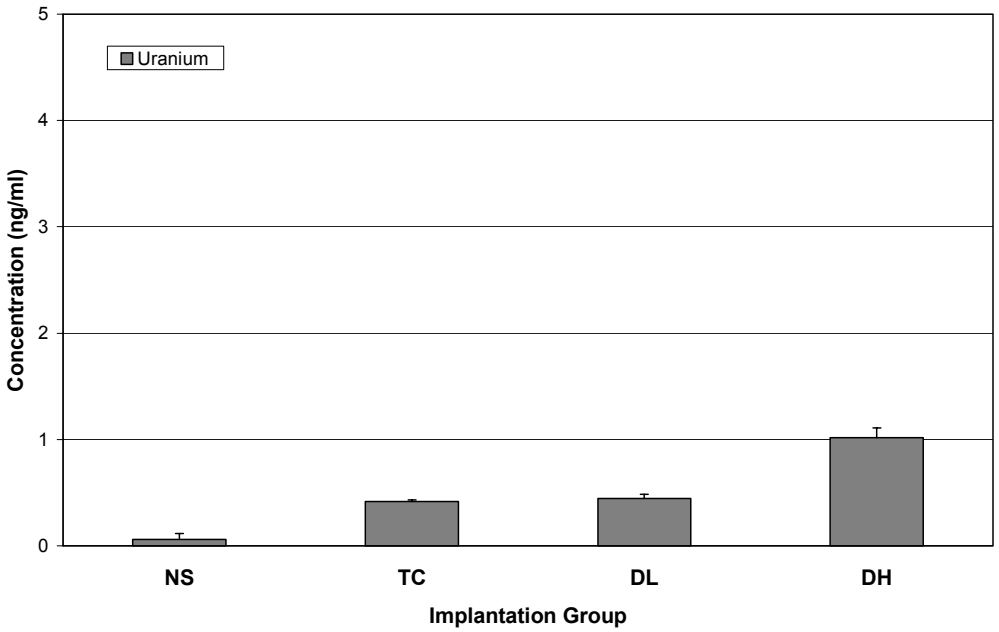


Figure 40. Serum Metal (Co, Ni, W) Concentrations: 6 Month Group. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

A



B

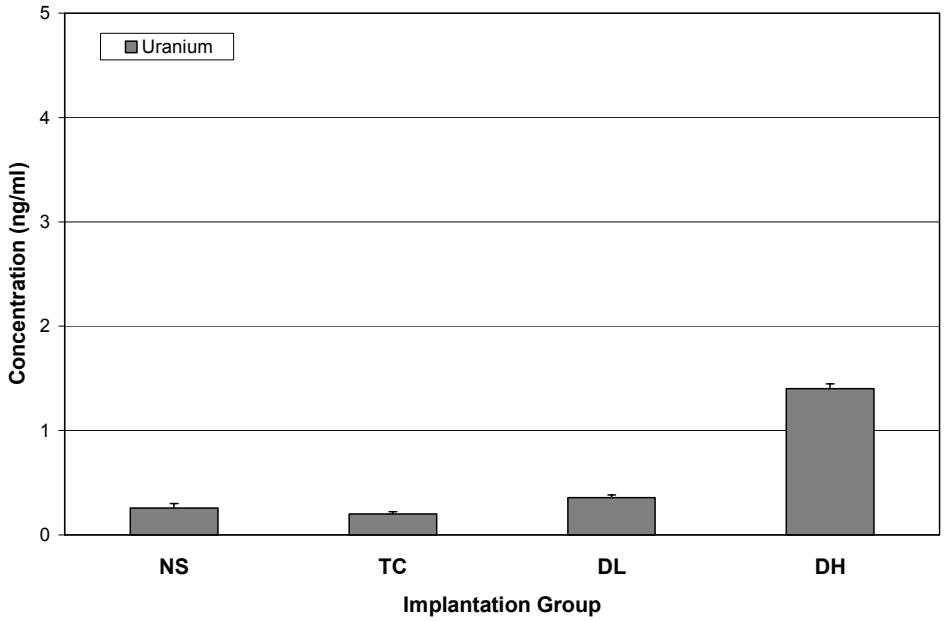
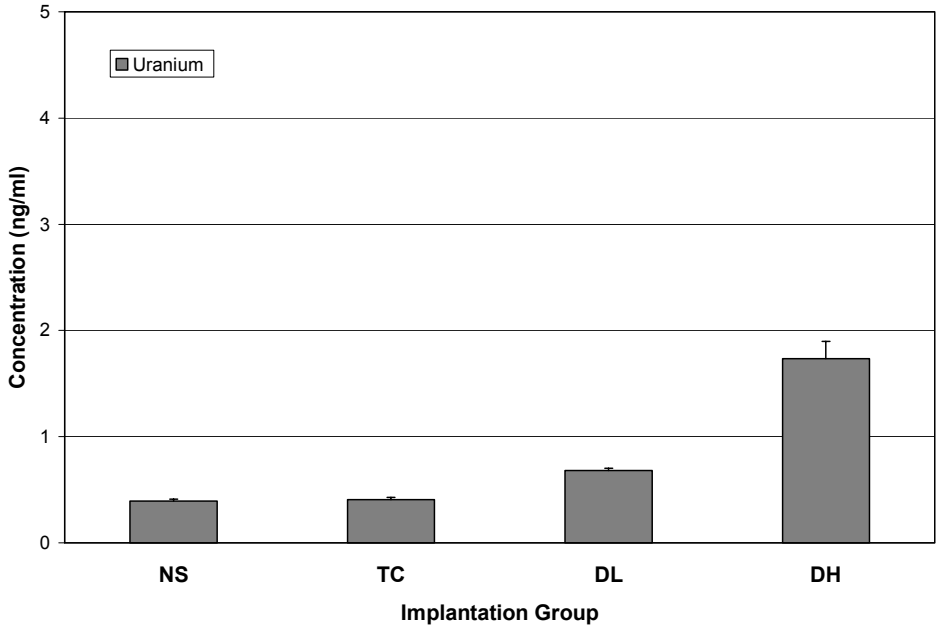


Figure 41. Serum Uranium Concentrations: 1 Month (Panel A) and 3 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

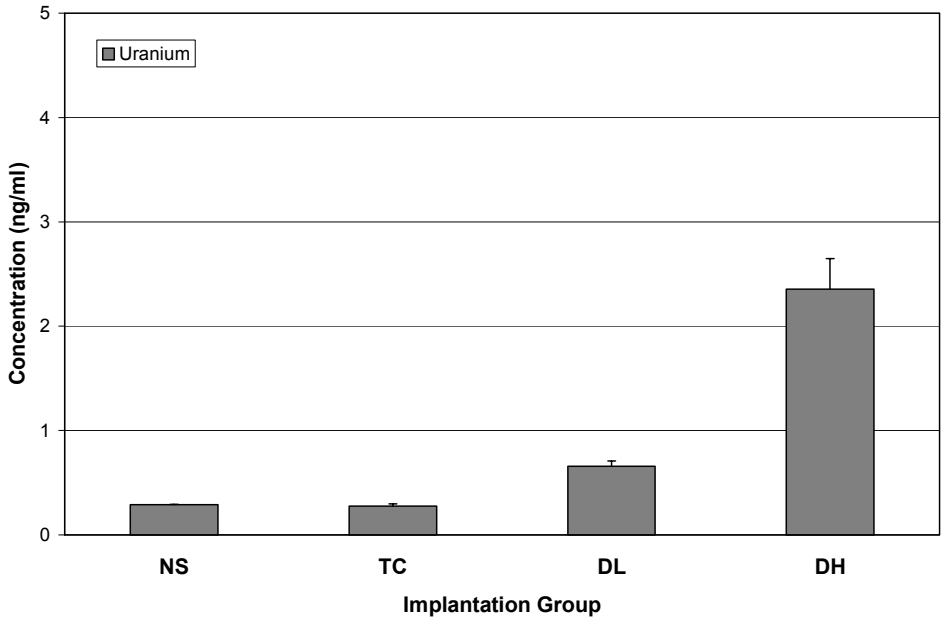


Figure 42. Serum Uranium Concentrations: 6 Month (Panel A) and 12 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

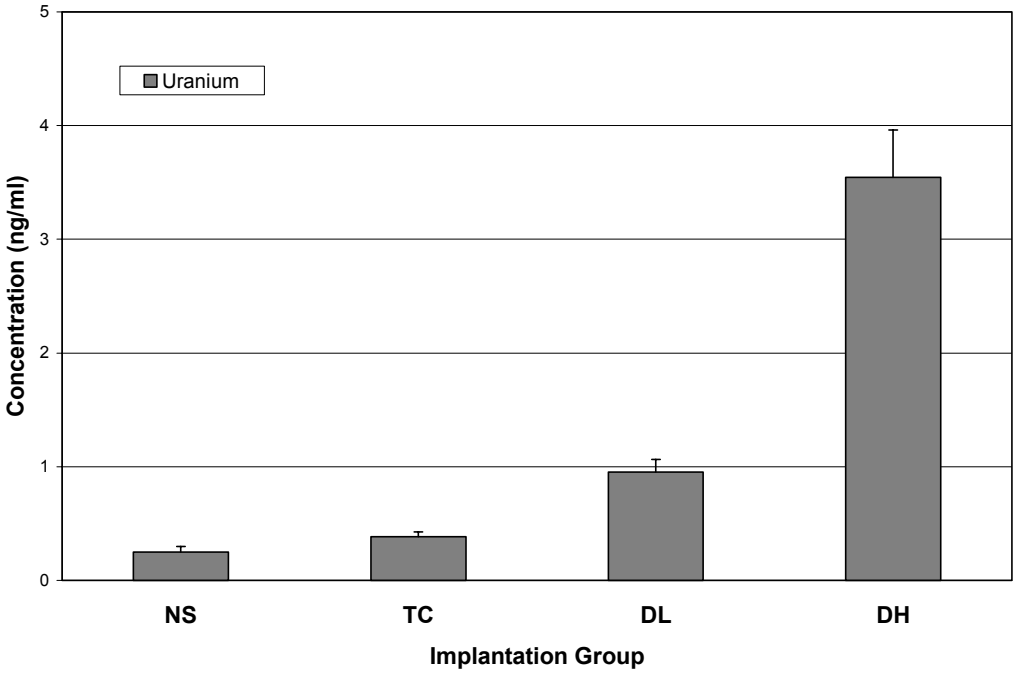
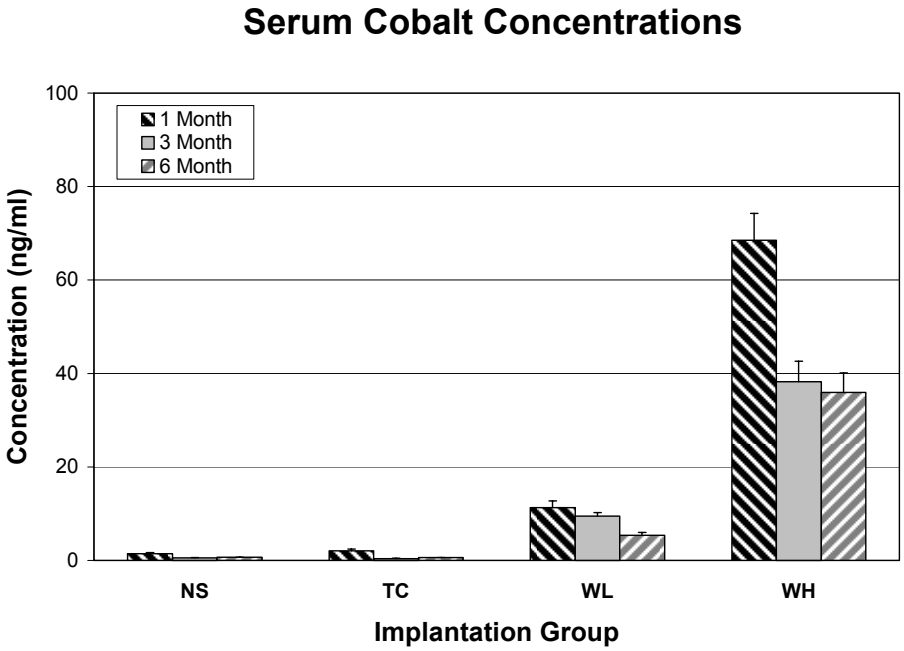


Figure 43. Serum Uranium Concentrations: 18 Month Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

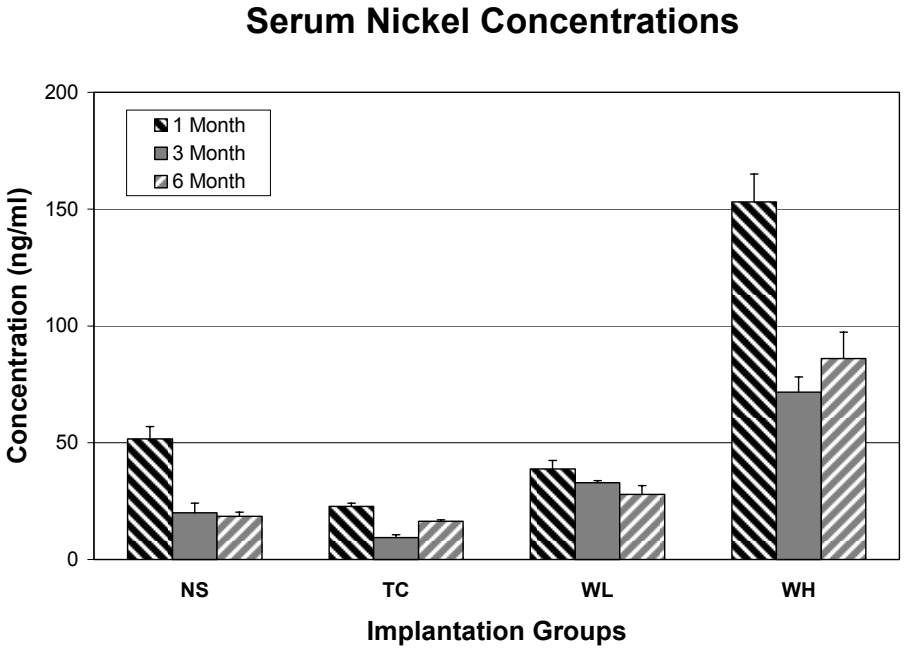
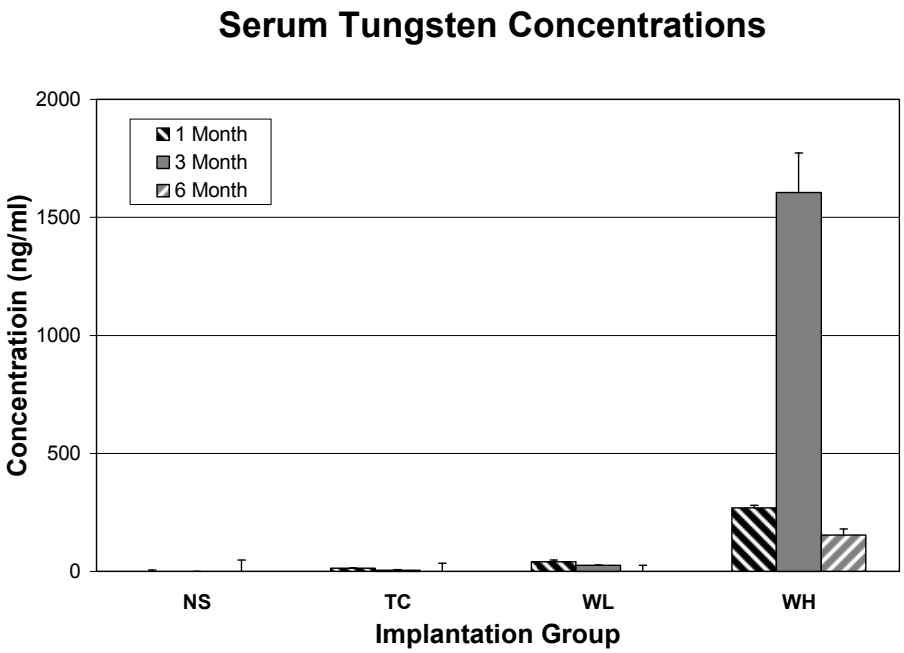


Figure 44. Serum Cobalt (Panel A) and Nickel (Panel B) Concentrations over Time. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

A



B

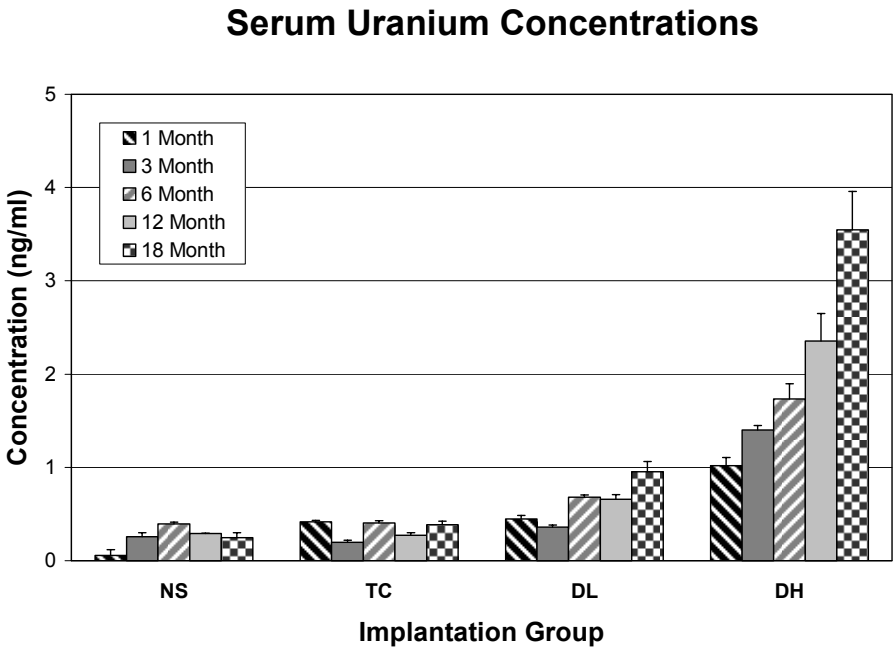
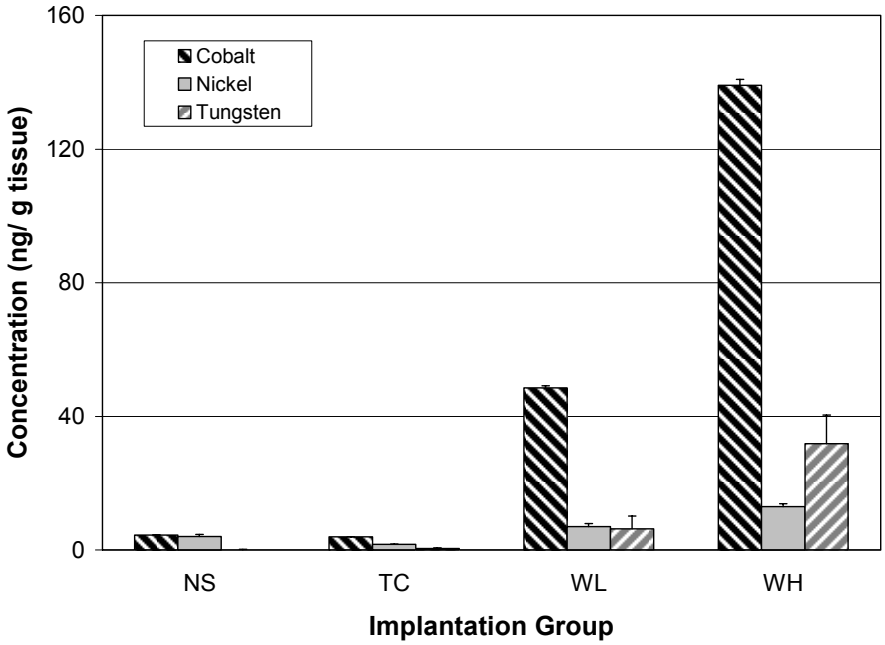


Figure 45. Serum Cobalt (Panel A) and Nickel (Panel B) Concentrations over Time. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose); DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

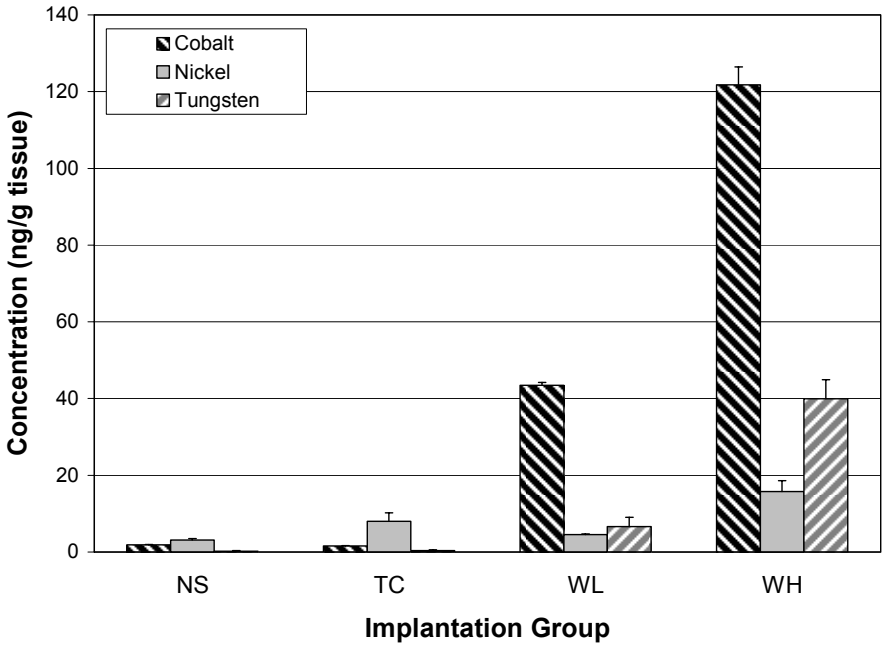


Figure 46. Liver Metal (Co, Ni, W) Concentrations: 1 Month (Panel A) and 3 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

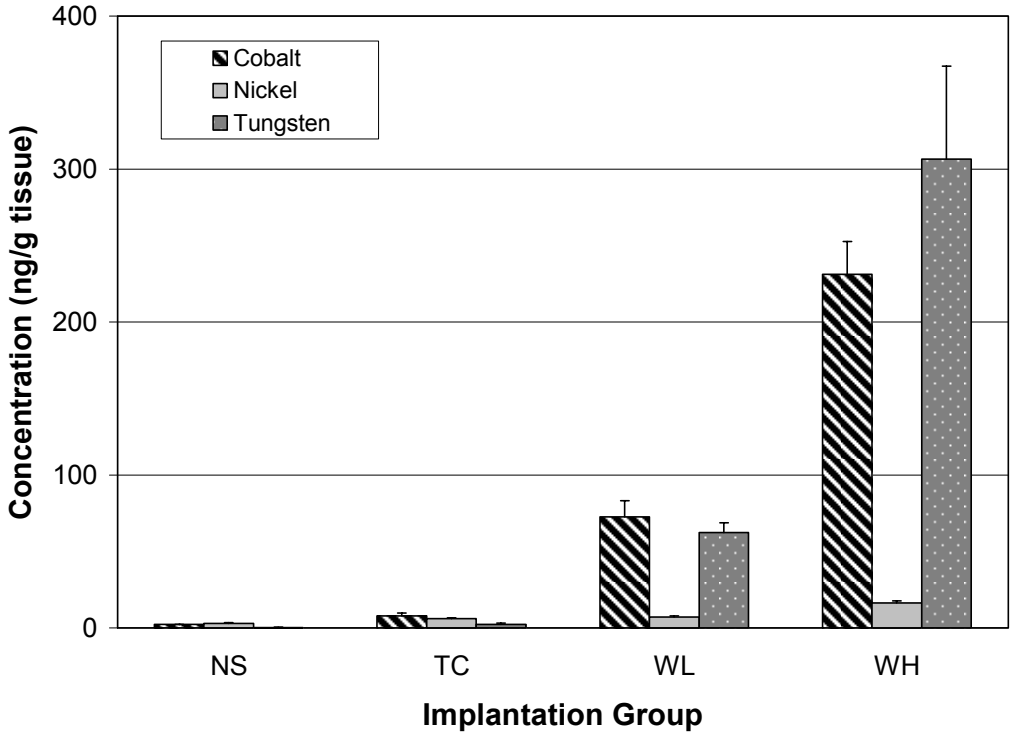
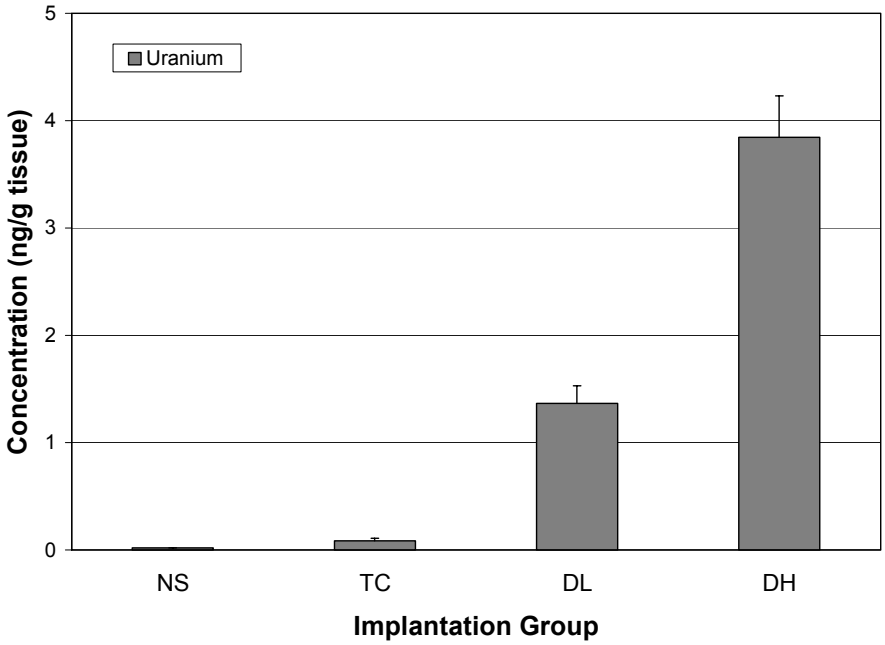


Figure 47. Liver Metal (Co, Ni, W) Concentrations: 6 Month Group. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

A



B

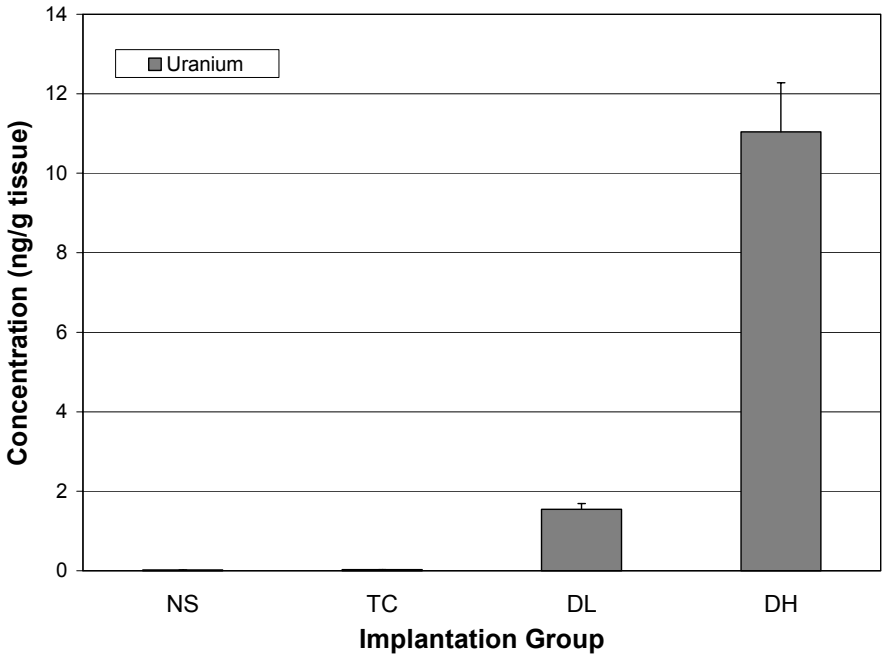
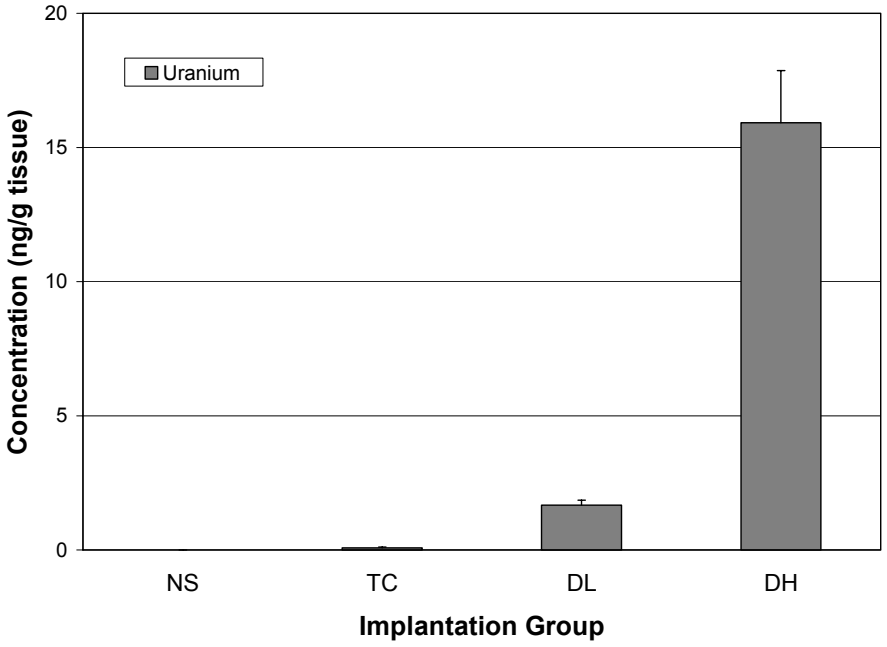


Figure 48. Liver Uranium Concentrations: 1 Month (Panel A) and 3 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

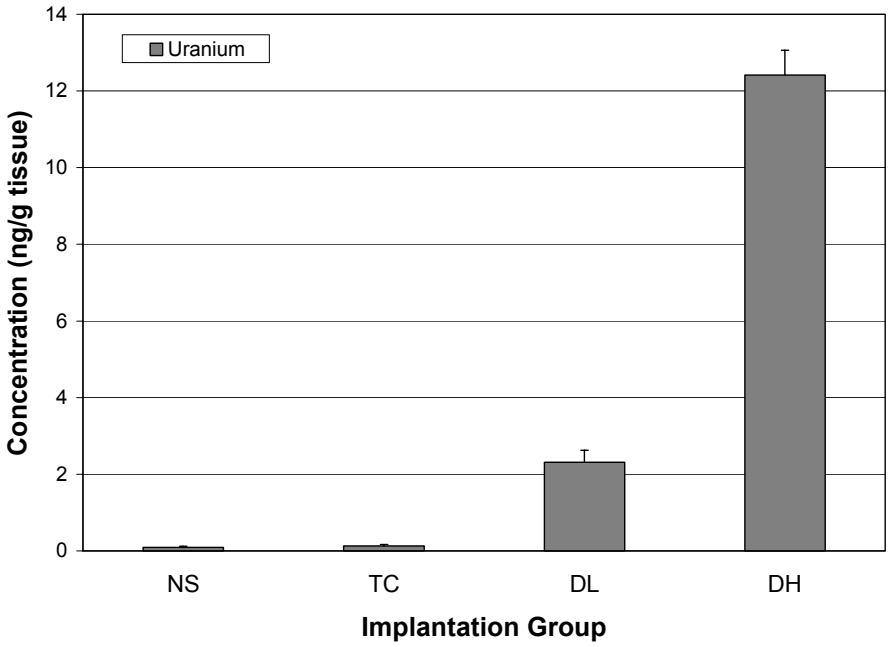


Figure 49. Liver Uranium Concentrations: 6 Month (Panel A) and 12 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH-depleted uranium (high dose).

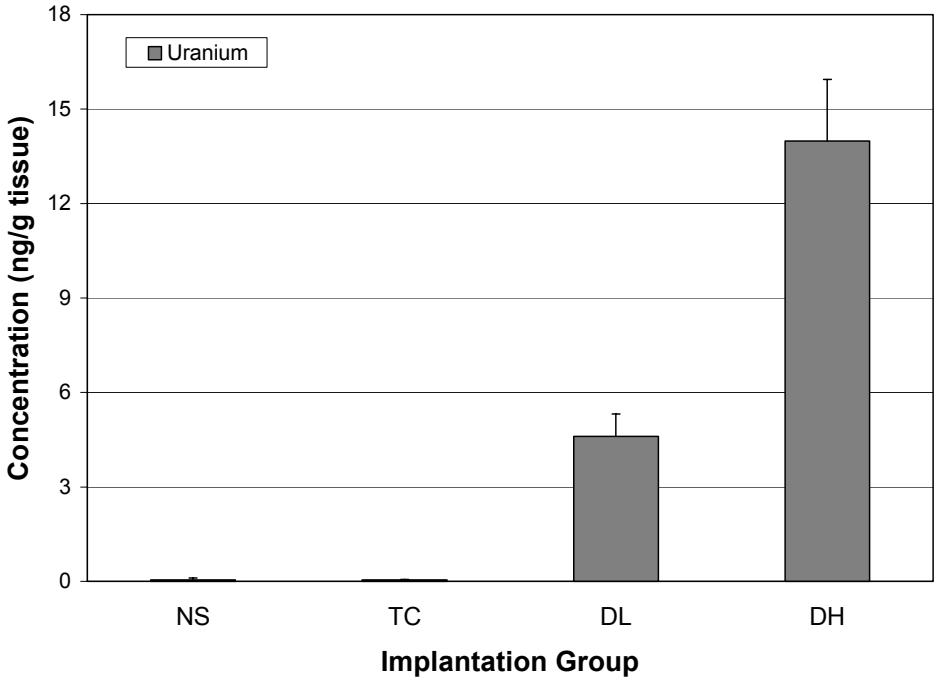
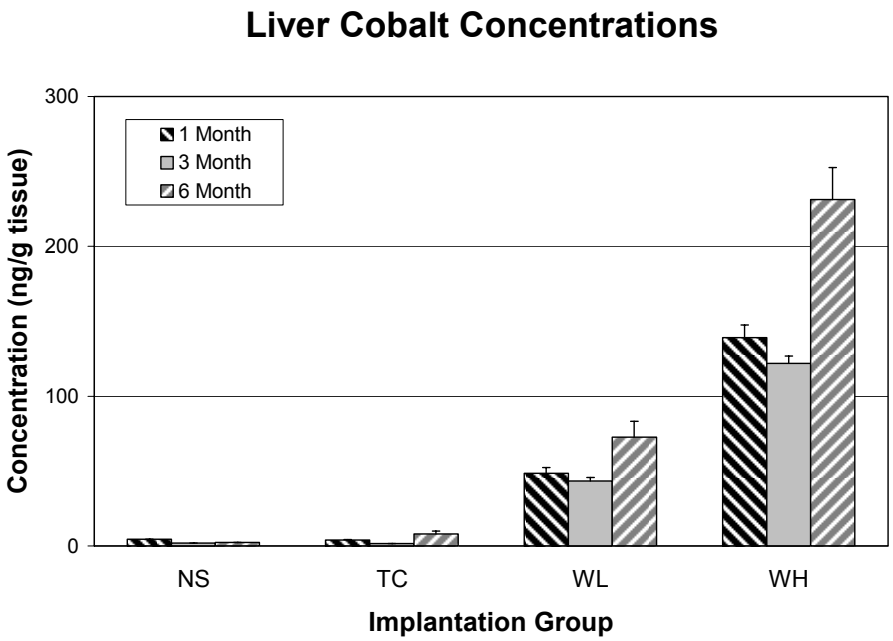


Figure 50. Liver Uranium Concentrations: 18 Month Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

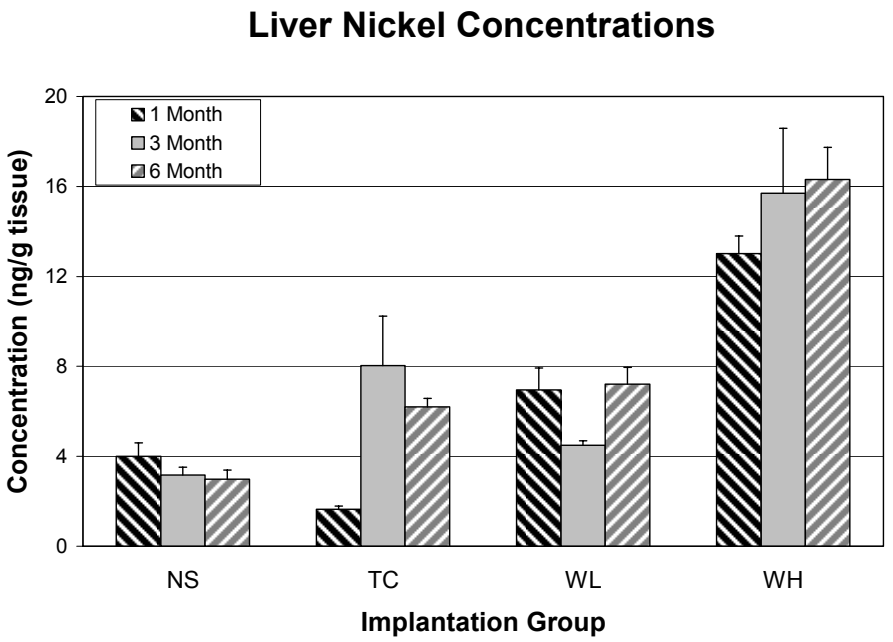
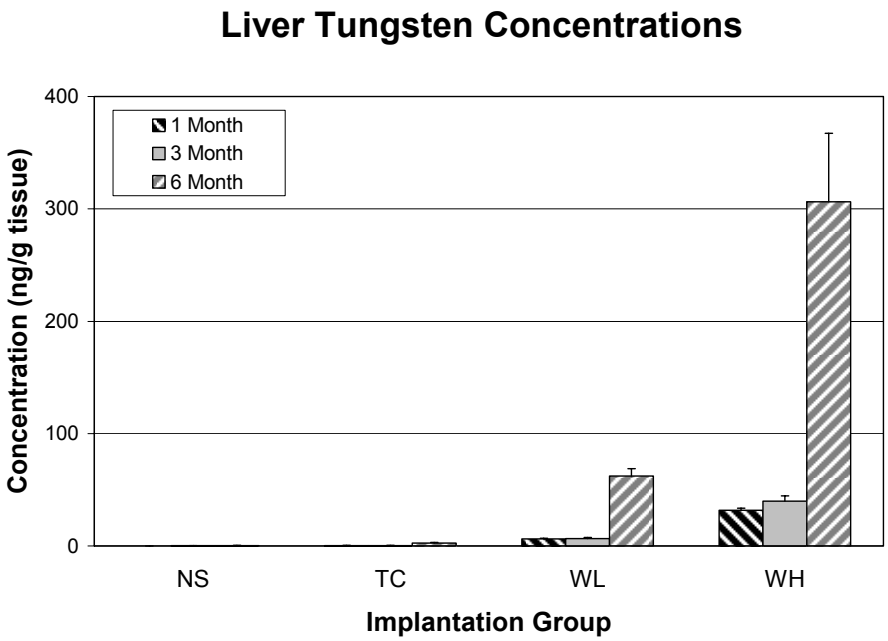


Figure 51. Liver Cobalt (Panel A) and Nickel (Panel B) Concentrations over Time. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

A



B

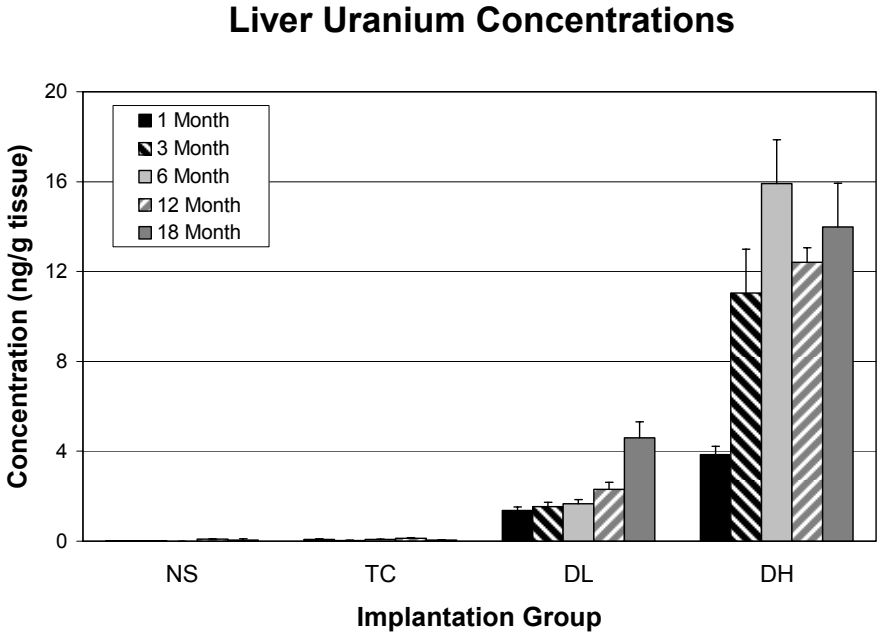
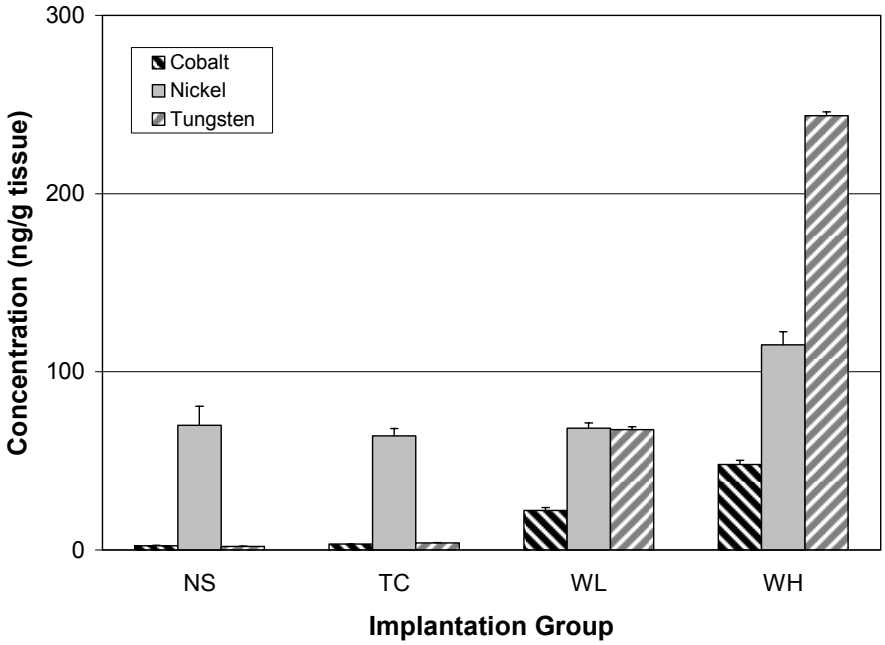


Figure 52. Liver Tungsten (Panel A) and Uranium (Panel B) Concentrations over Time. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose); DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

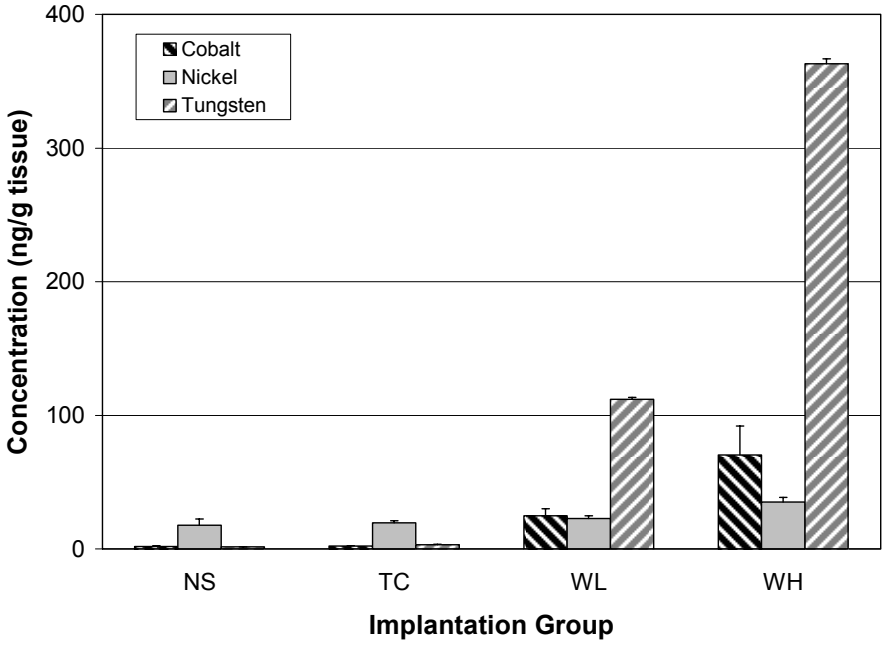


Figure 53. Spleen Metal (Co, Ni, W) Concentrations: 1 Month (Panel A) and 3 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

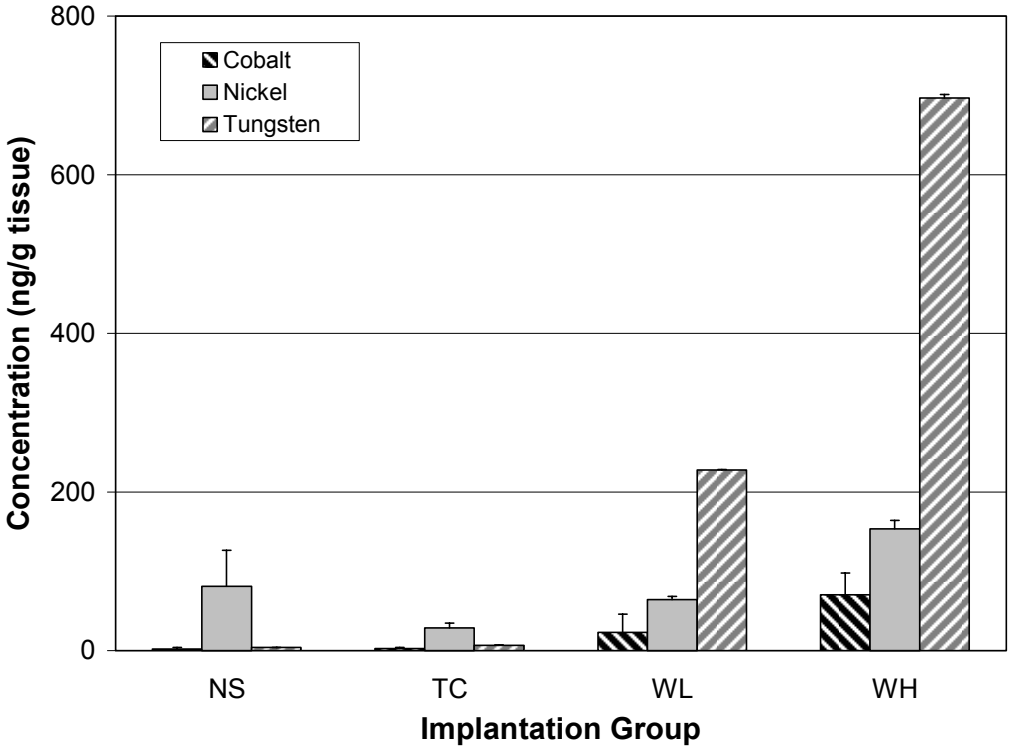
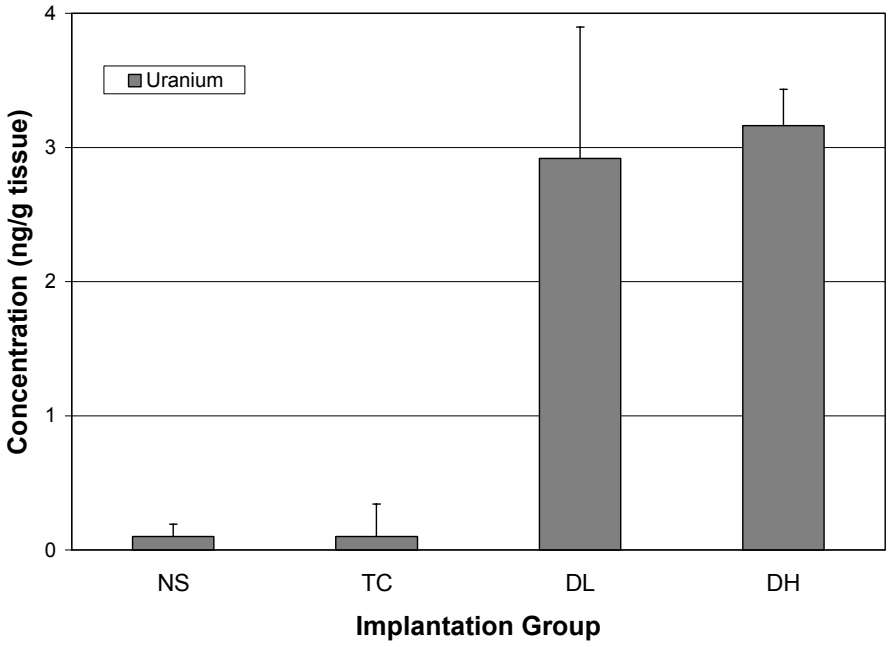


Figure 54. Spleen Metal (Co, Ni, W) Concentrations: 6 Month Group. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

A



B

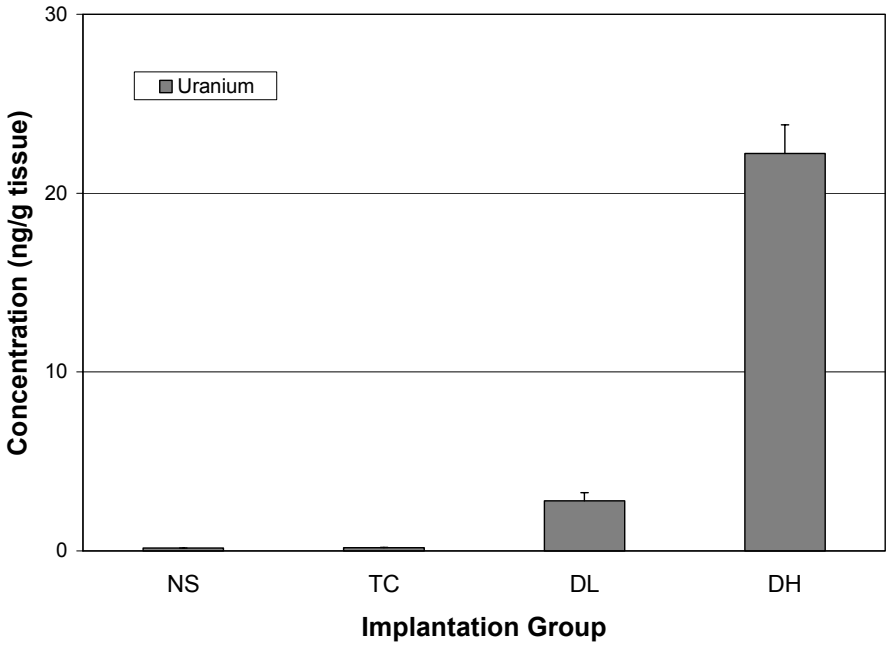
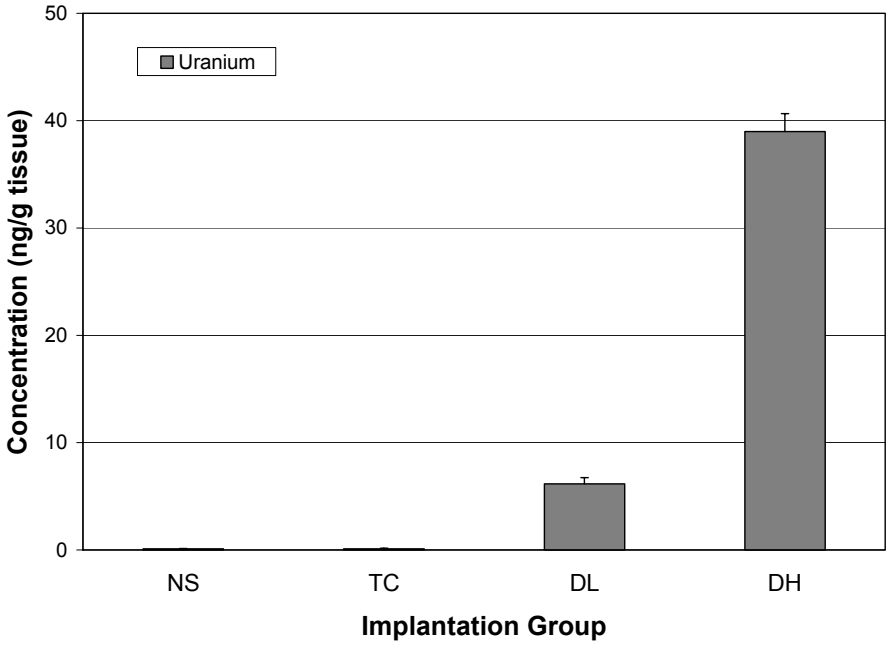


Figure 55. Spleen Uranium Concentrations: 1 Month (Panel A) and 3 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

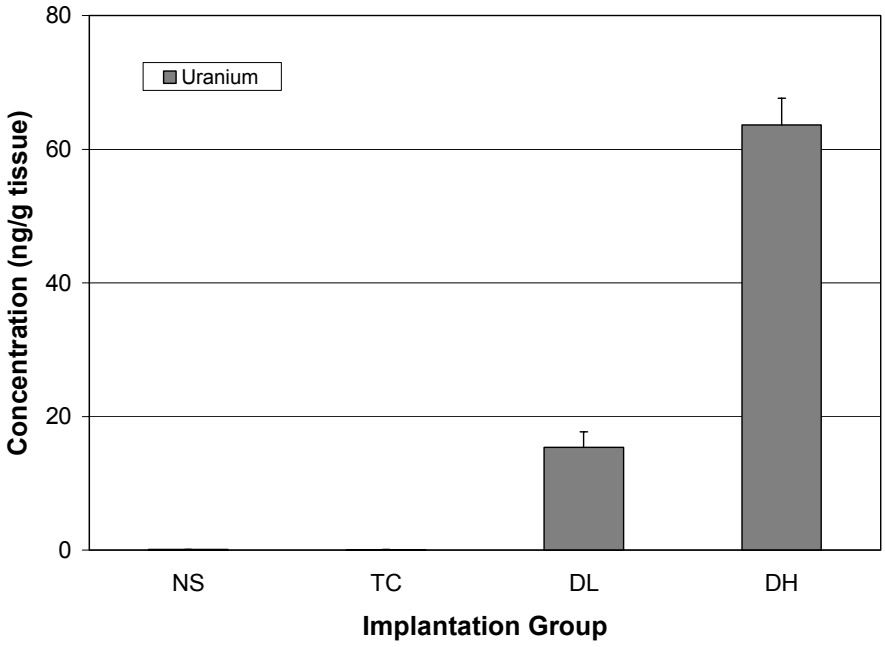


Figure 56. Spleen Uranium Concentrations: 6 Month (Panel A) and 12 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

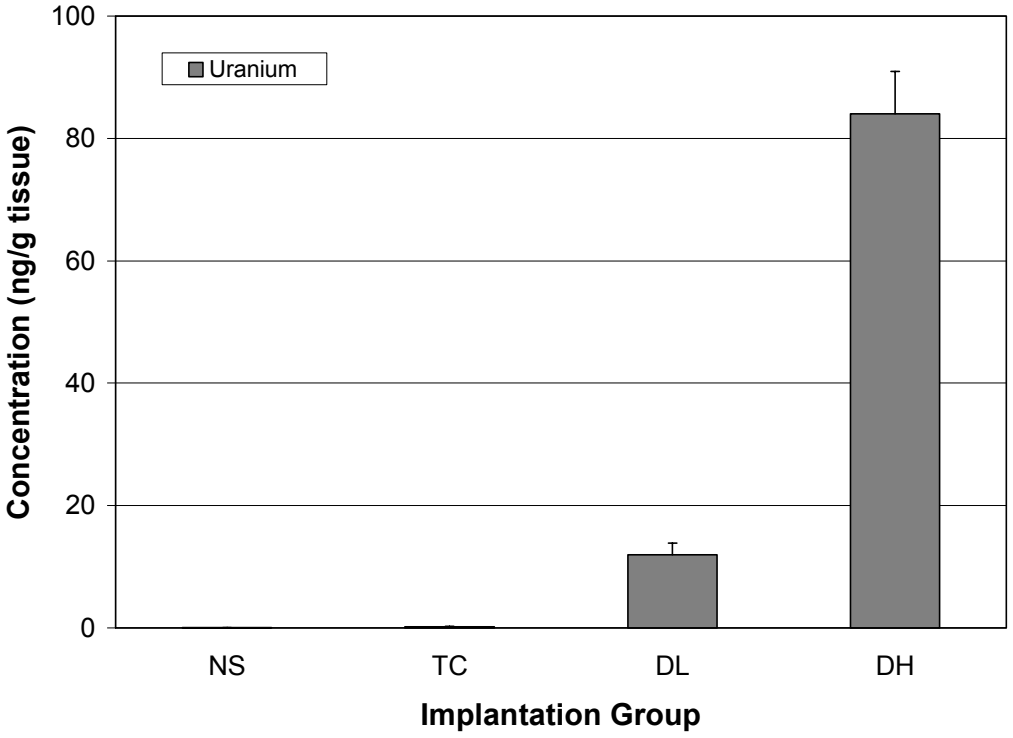
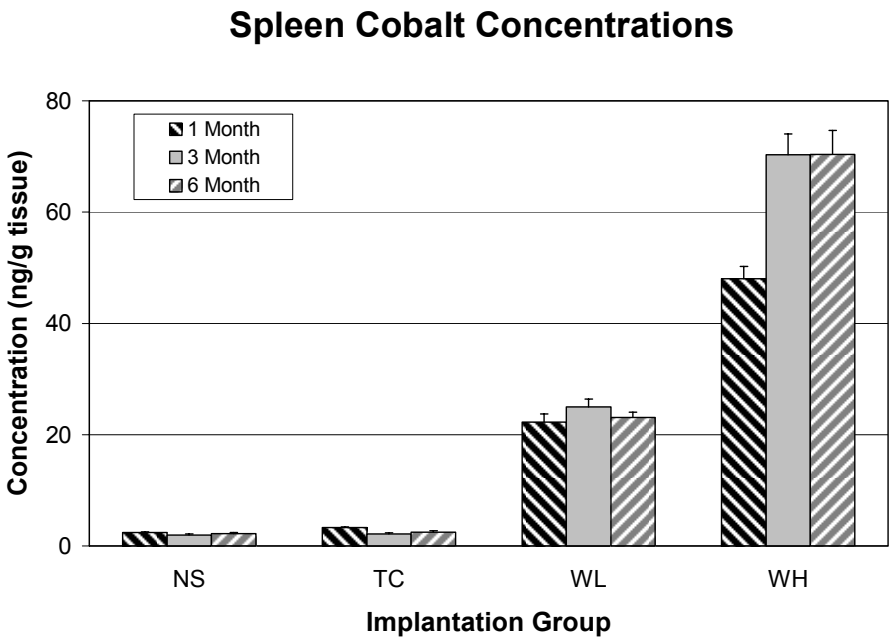


Figure 57. Spleen Uranium Concentrations: 18 Month Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

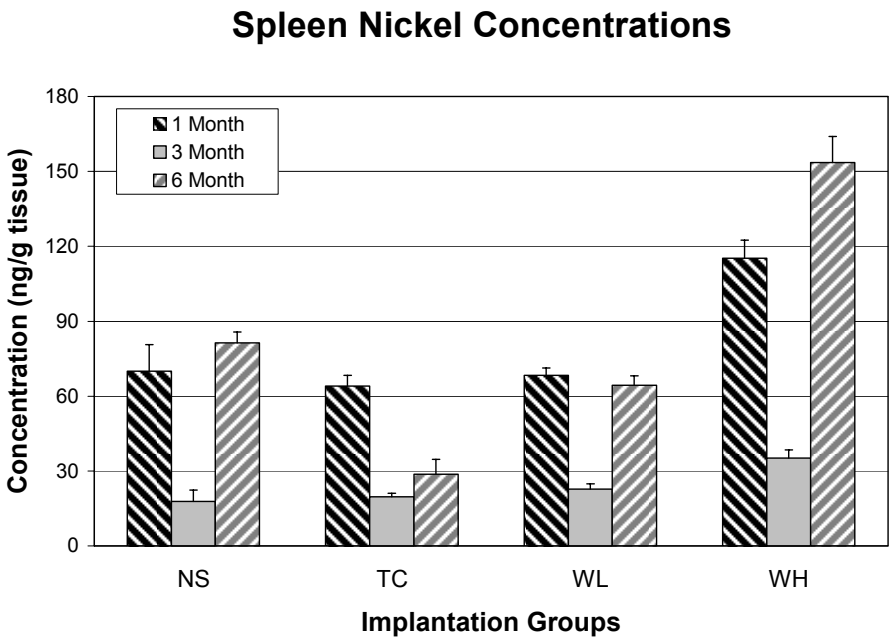
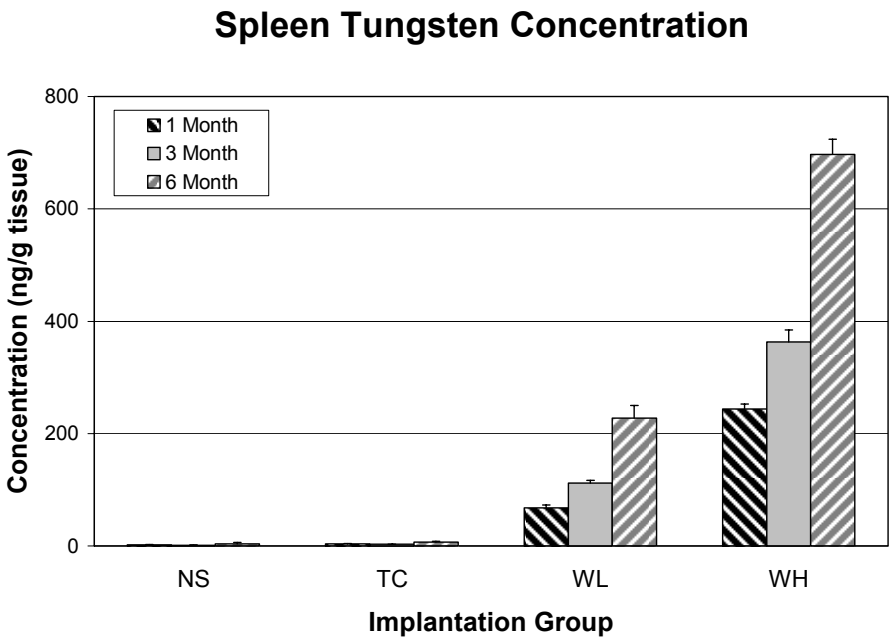


Figure 58. Spleen Cobalt (Panel A) and Nickel (Panel B) Concentrations over Time. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

A



B

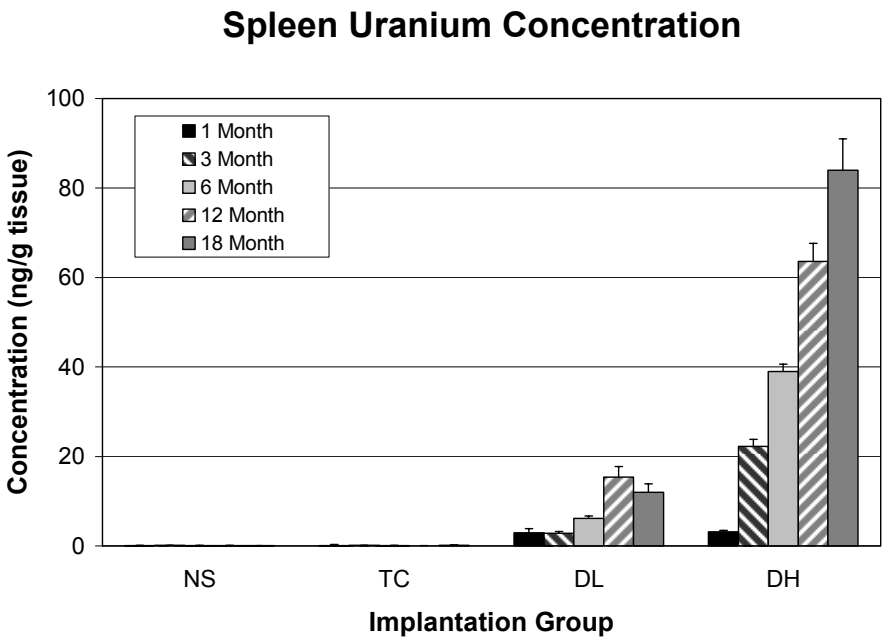
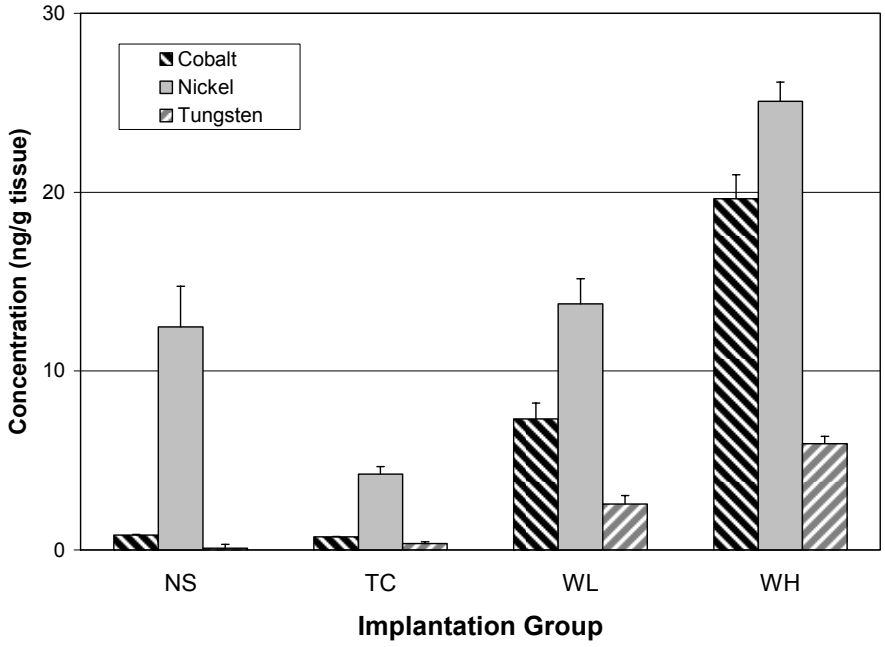


Figure 59. Kidney Cobalt (Panel A) and Nickel (Panel B) Concentrations over Time. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose); DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

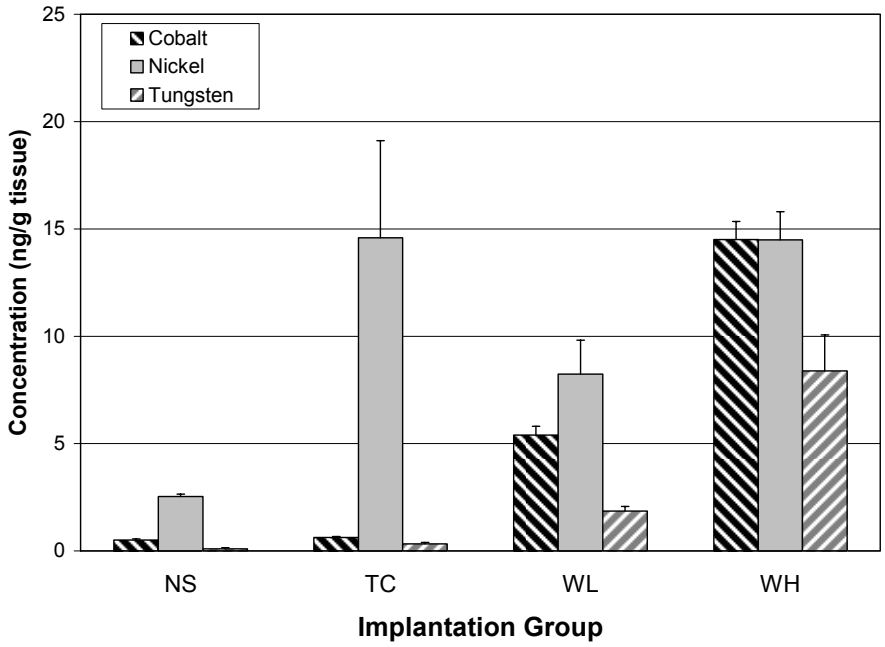


Figure 60. Muscle Metal (Co, Ni, W) Concentrations: 1 Month (Panel A) and 3 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

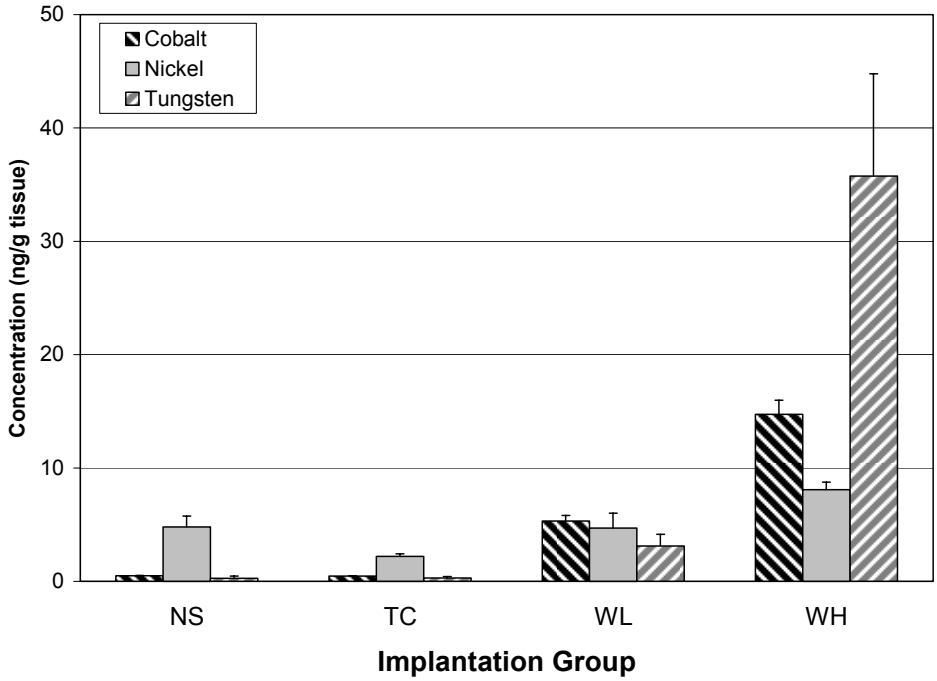
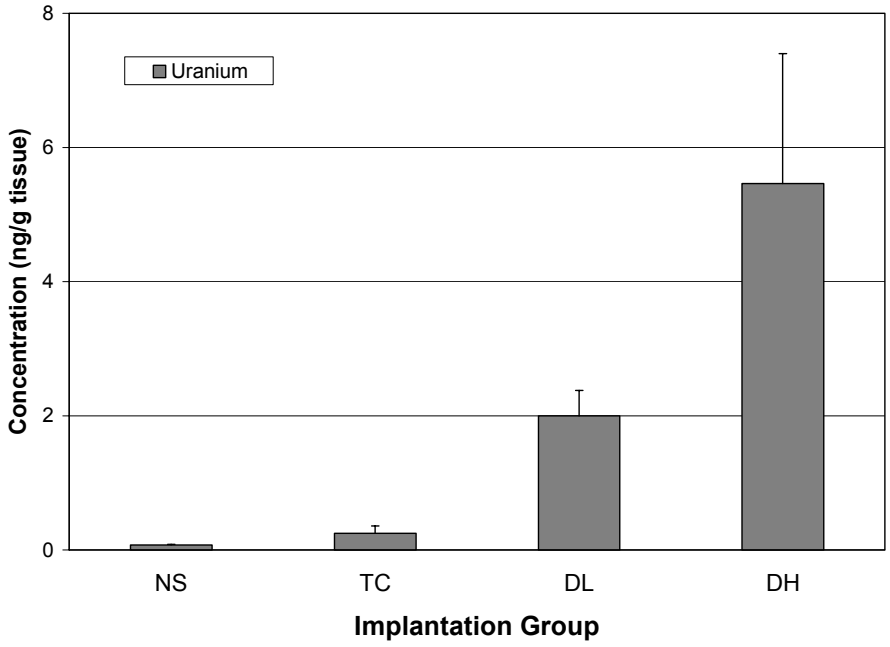


Figure 61. Muscle Metal (Co, Ni, W) Concentrations: 6 Month Group. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

A



B

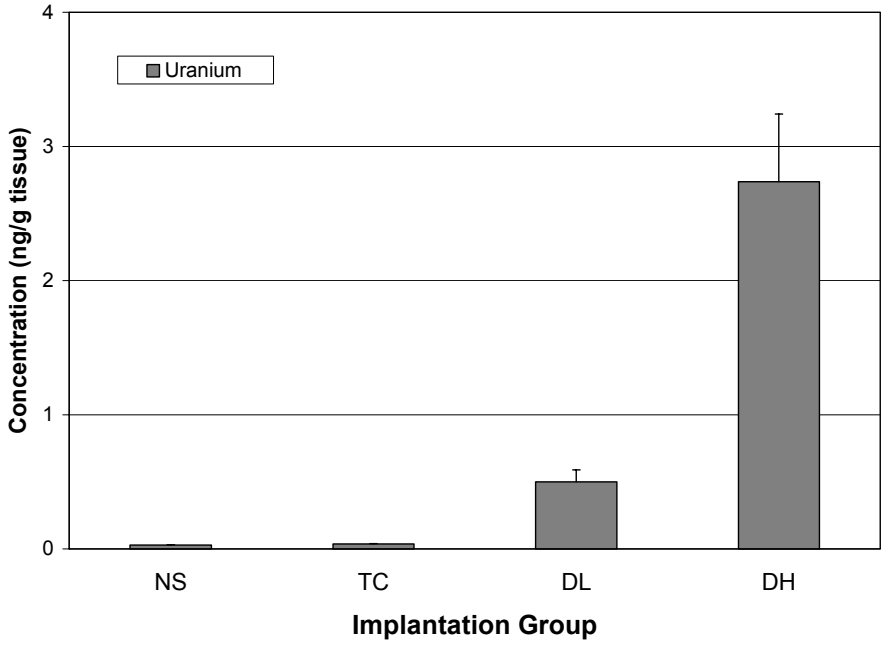
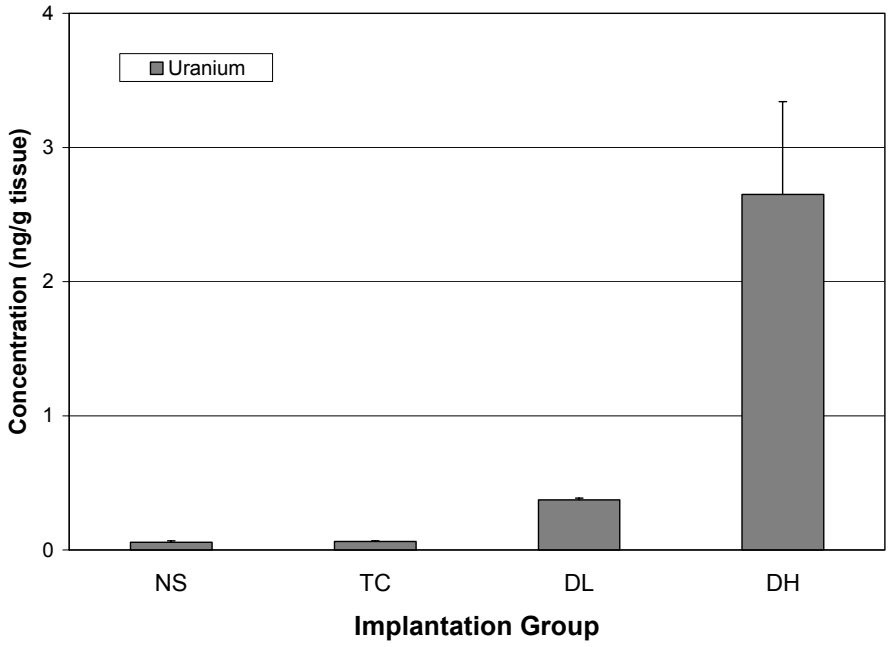


Figure 62. Muscle Uranium Concentrations: 1 Month (Panel A) and 3 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

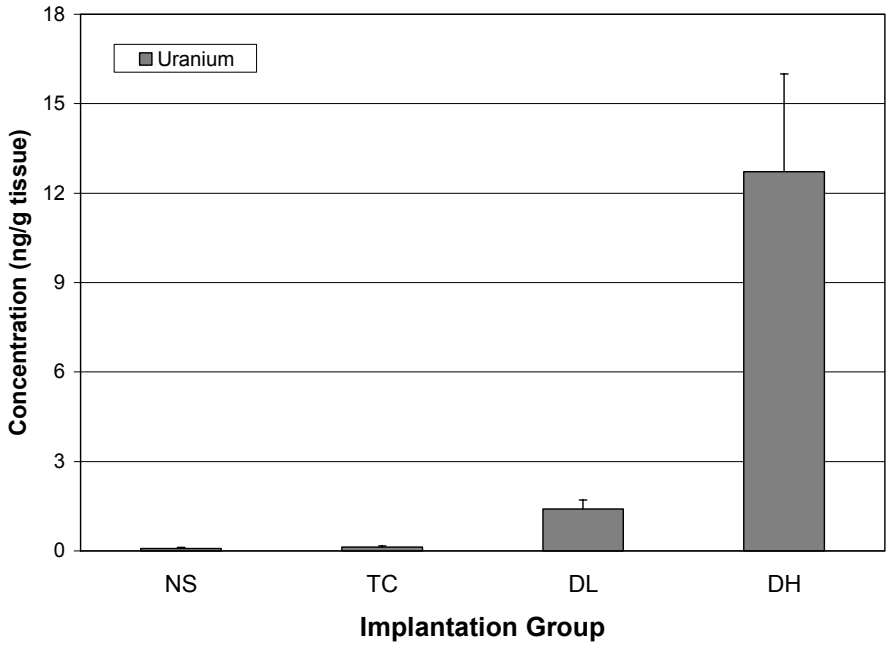


Figure 63. Muscle Uranium Concentrations: 6 Month (Panel A) and 12 Month (Panel B) Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

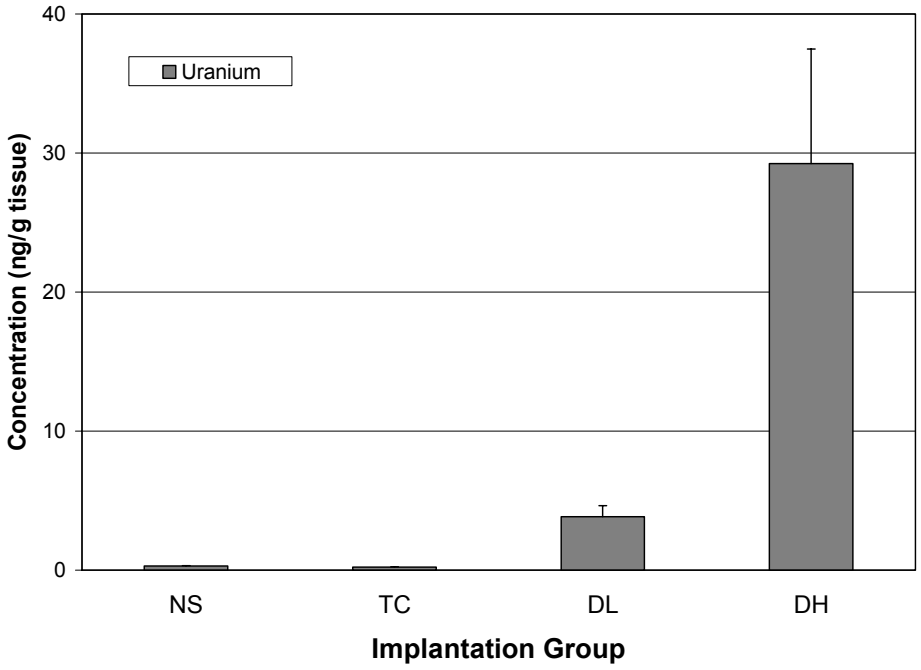
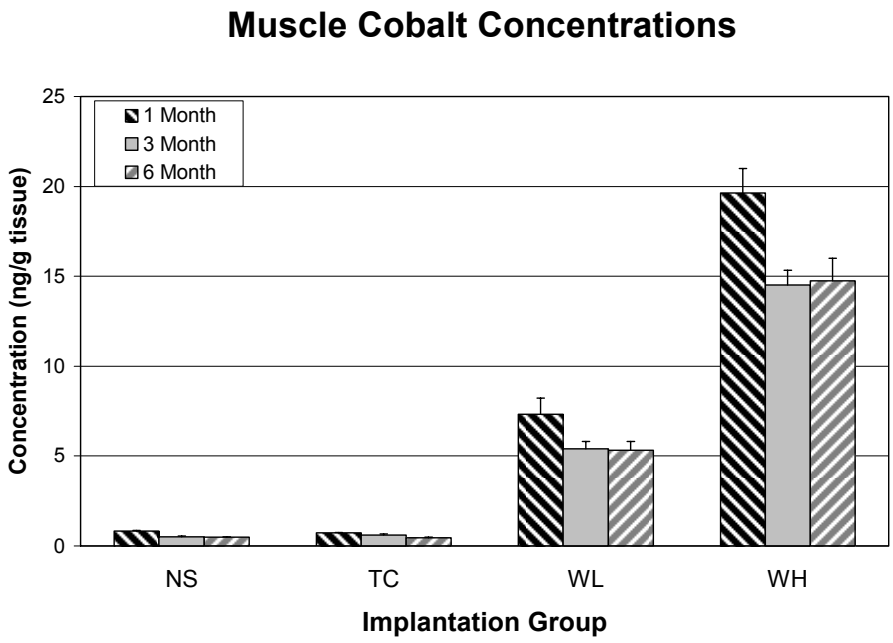


Figure 64. Muscle Uranium Concentrations: 18 Month Groups. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; DL-depleted uranium (low dose); DH- depleted uranium (high dose).

A



B

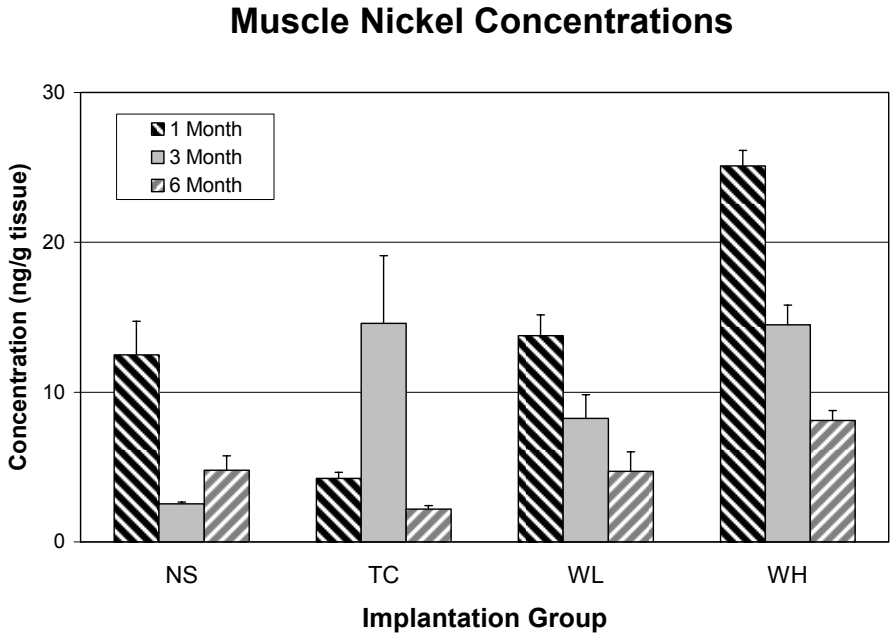
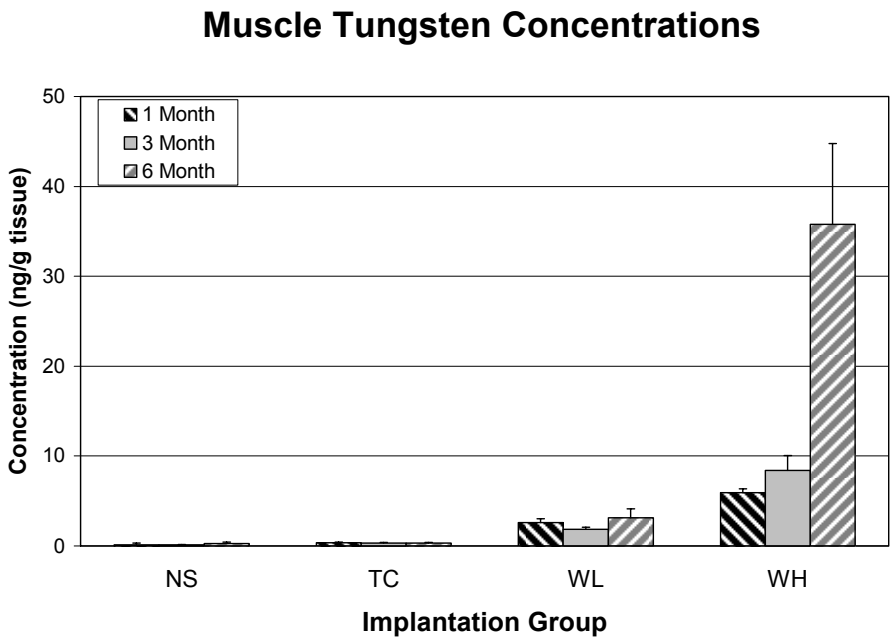


Figure 65. Muscle Cobalt (Panel A) and Nickel (Panel B) Concentrations over Time. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose).

A



B

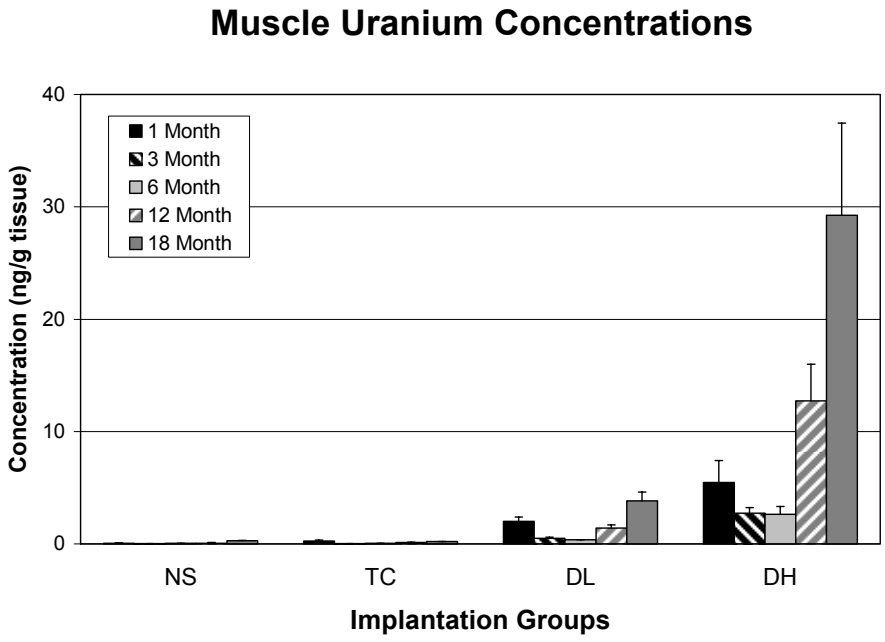


Figure 66. Muscle Tungsten (Panel A) and Uranium (Panel B) Concentrations over Time. Data are the mean of 10 independent samples. Error bars represent standard error of the mean. NS-non-surgical; TC-tantalum control; WL-tungsten alloy (low dose); WH-tungsten alloy (high dose); DL-depleted uranium (low dose); DH- depleted uranium (high dose).

Figure 67. Metaphase Chromosome Spread from Rat Lymphocytes

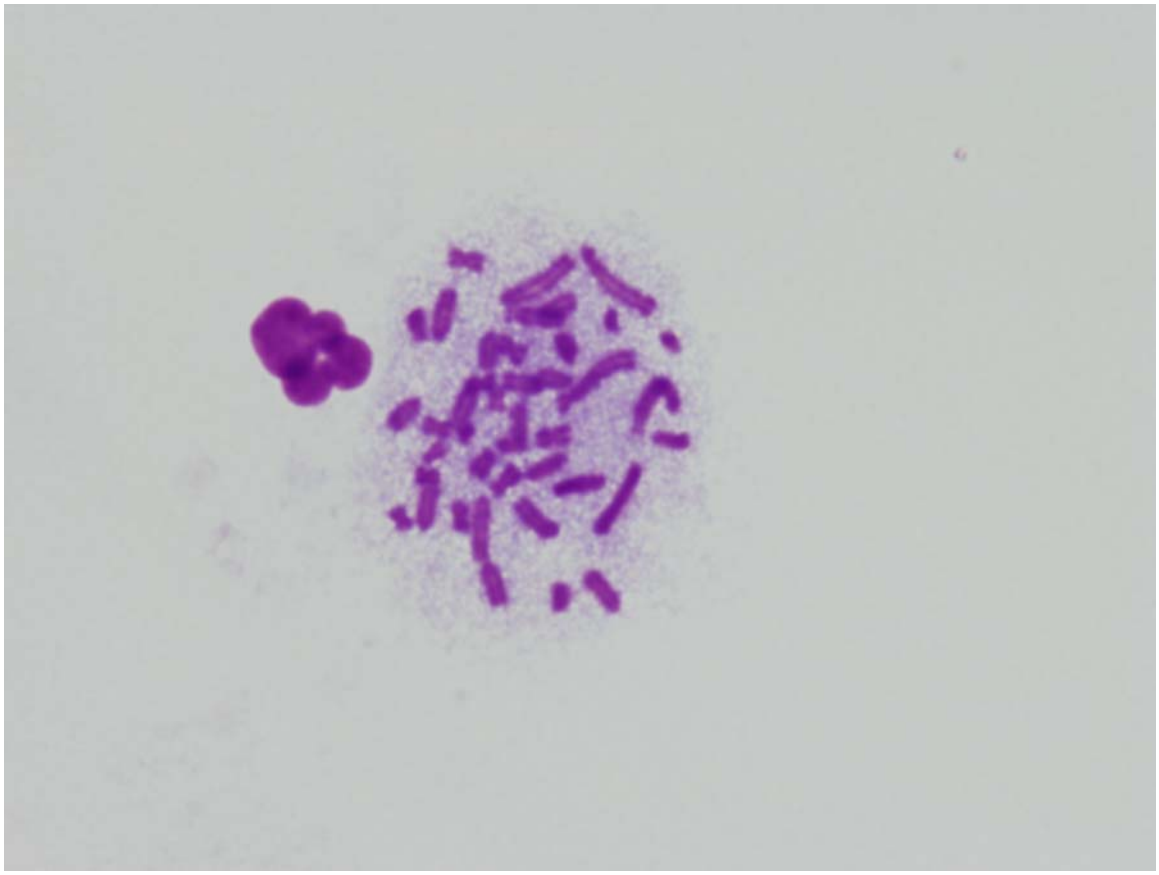


TABLE 28. Serum and urine mutagenicity

Time (months)	Experimental Group	Mutagenicity (revertants/ul sample)
	Serum	
6 month	Non-Surgical	0.171
	Tantalum	0.188
	DU-Low	0.154
	DU-High	0.129
	WA-Low	0.133
	WA-High	0.117
	Nickel	0.165
12 month	Non-Surgical	0.171
	Tantalum	0.188
	DU-Low	0.154
	DU-High	0.129
18 month	Non-Surgical	0.195 ± 0.024
	Tantalum	0.159 ± 0.032
	DU-Low	0.188 ± 0.049
	DU-High	0.121 ± 0.088
	Urine	
6 month	Non-Surgical	29.42 ± 10.08
	Tantalum	27.85 ± 11.80
	DU-Low	45.83 ± 10.78
	DU-High	115.18 ± 15.35
	WA-Low	58.04 ± 12.31
	WA-High	94.37 ± 13.95
	Nickel	44.75 ± 10.63
12 month	Non-Surgical	35.92 ± 8.14
	Tantalum	33.57 ± 9.60
	DU-Low	83.28 ± 10.34
	DU-High	257.53 ± 32.81

Table 29. Immune Organ Cellularities for 1-Month Rodents

1 Month	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High
Spleen Cellularity (10e8 cells/g tissue)	25.9 ± 1.2	21.9 ± 1.1	24.7 ± 1.4	24.1 ± 1.5	19.3 ± 0.9 *	17.4 ± 1.0 *
Thymus Cellularity (10e8 cells/g tissue)	32.2 ± 1.3	22.6 ± 1.4	21.4± 1.7 *	27.4 ± 2.3	22.6 ± 1.1 *	22.4 ± 1.1 *
Bone Marrow Cellularity (10e7 cells/femur)	9.3 ± 0.6	11.2 ± 1.2	8.8 ± 0.5	8.2 ± 0.6	10.0 ± 0.5	9.0 ± 0.4

Data are expressed as mean +/- standard error of the mean of 15 replicates. * represents a significant difference from control at $P<0.05$ using Students t-test.

Table 30. Immune Organ Cellularities for 3-Month Rodents

3 Month	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High
Spleen Cellularity (10e8 cells/g tissue)	24.2 ± 1.5	22.7 ± 1.2	20.5 ± 0.8 *	21.8 ± 1.0	20.2 ± 1.0 *	21.4 ± 1.1
Thymus Cellularity (10e8 cells/g tissue)	19.3 ± 0.7	23.1 ± 1.3	21.6± 1.7	25.2 ± 2.2 *	18.1 ± 1.0	22.9 ± 1.6
Bone Marrow Cellularity (10e7 cells/femur)	10.2 ± 0.6	9.5 ± 0.7	8.3 ± 0.4 *	8.0 ± 0.4 *	8.6 ± 0.4 *	11.5 ± 0.5

Data are expressed as mean +/- standard error of the mean of 15 replicates. * represents a significant difference from control at $P<0.05$ using Students t-test.

Table 31. Immune Organ Cellularities for 6-Month Rodents

6 Month	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High
Spleen Cellularity (10e8 cells/g tissue)	33.2 ± 1.4	29.9 ± 1.6	22.0 ± 1.2 *	26.9 ± 1.7 *	31.0 ± 1.6	27.2 ± 1.3 *
Thymus Cellularity (10e8 cells/g tissue)	10.6 ± 1.0	12.9 ± 1.4	7.1 ± 0.7 *	15.3 ± 1.2 *	8.4 ± 0.4	11.2 ± 0.5
Bone Marrow Cellularity (10e7 cells/femur)	13.0 ± 0.6	12.3 ± 0.5	7.8 ± 0.4 *	11.5 ± 0.5	11.6 ± 0.9	13.4 ± 0.8

Data are expressed as mean +/- standard error of the mean of 15 replicates. * represents a significant difference from control at $P<0.05$ using Students t-test.

Table 32. Immune Organ Cellularities for 12-Month Rodents

12 Month	Non-Surgical	Tantalum	DU-Low	DU-High
Spleen Cellularity (10e8 cells/g tissue)	29.7 ± 1.2	31.3 ± 1.8	31.2 ± 1.4	27.7 ± 1.7
Thymus Cellularity (10e8 cells/g tissue)	8.5 ± 1.0	6.6 ± 1.0	4.6 ± 0.7 *	8.3 ± 0.7
Bone Marrow Cellularity (10e7 cells/femur)	14.8 ± 1.2	13.6 ± 0.7	12.7 ± 1.0	10.7 ± 0.7 *

Data are expressed as mean +/- standard error of the mean of 15 replicates. * represents a significant difference from control at $P<0.05$ using Students t-test.

TABLE 33. Flow Cytometric Analysis – 1 Month Animals

Thymus

Group	CD4-CD8-	CD4+CD8-	CD4-CD8+	CD4+CD8+
NS	3.32 ± 0.14	6.68 ± 0.31	4.70 ± 0.09	85.30 ± 0.43
TC	4.04 ± 0.29	6.92 ± 0.28	4.55 ± 0.32	84.50 ± 0.72
DL	3.05 ± 0.13	5.77 ± 0.42	4.17 ± 0.13	87.01 ± 0.41
DH	3.59 ± 0.15	6.17 ± 0.21	4.46 ± 0.14	85.78 ± 0.39
WL	3.32 ± 0.12	6.14 ± 0.18	4.39 ± 0.19	86.15 ± 0.31
WH	3.31 ± 0.10	5.46 ± 0.20	4.66 ± 0.10	86.56 ± 0.28

Blood

Group	Cytotoxic T cell	T helper	Naïve CD4	Activated CD4	Naïve CD8	Activated CD8
NS	11.14 ± 1.19	18.61 ± 2.04	13.15 ± 1.46	6.30 ± 0.75	13.50 ± 1.95	3.24 ± 1.08
TC	12.71 ± 0.95	21.53 ± 1.50	13.66 ± 0.91	8.76 ± 0.65	12.09 ± 1.16	1.01 ± 0.07
DL	10.57 ± 0.61	18.16 ± 0.88	9.70 ± 0.42	9.22 ± 0.68	9.94 ± 0.55	1.07 ± 0.09
DH	11.04 ± 1.09	18.22 ± 1.98	10.10 ± 1.03	7.12 ± 0.77	9.37 ± 0.74	1.11 ± 0.15
WL	10.52 ± 0.74	17.29 ± 1.15	10.14 ± 1.08	8.48 ± 0.59	9.71 ± 0.81	1.19 ± 0.19
WH	7.50 ± 0.39 *	12.75 ± 0.76 *	5.75 ± 0.70 *	6.98 ± 1.03	6.74 ± 0.35 *	0.90 ± 0.07

Spleen

Group	T cells	B cells	Cytotoxic T cells	NK cells	T helper	Putative Monocytes
NS	27.26 ± 0.45	45.30 ± 0.64	9.46 ± 0.33	9.32 ± 0.25	12.88 ± 0.19	4.55 ± 0.25
TC	25.45 ± 0.45	46.77 ± 0.99	18.30 ± 0.56	6.75 ± 0.56 *	11.26 ± 0.31	3.24 ± 0.19
DL	25.47 ± 0.32	43.77 ± 1.83	17.55 ± 0.37	2.29 ± 0.18 *	11.12 ± 0.29	3.33 ± 0.22
DH	26.55 ± 0.79	42.41 ± 1.19	18.31 ± 0.49	4.73 ± 0.39 *	11.78 ± 0.18	3.87 ± 0.19
WL	25.98 ± 0.44	41.05 ± 0.65 *	18.74 ± 0.50	4.64 ± 0.41 *	11.70 ± 0.32	3.90 ± 0.13
WH	25.90 ± 0.50	39.10 ± 0.59 *	18.30 ± 0.27	3.87 ± 0.26 *	12.00 ± 0.24	3.25 ± 0.11

* Statistically different from control using one-way ANOVA followed by Dunnett's test for group comparisons ($P<0.05$). Groups: NS (Non-surgical); TC (Tantalum control); DL (DU-low dose); DH (DU-high dose); WL (WA-low dose); WH (WA-high dose).

TABLE 34. Flow Cytometric Analysis – 3 Month Animals

Thymus

Group	CD4-CD8-	CD4+CD8-	CD4-CD8+	CD4+CD8+
NS	4.31 ± 0.42	7.10 ± 0.44	4.71 ± 0.24	83.88 ± 1.06
TC	3.69 ± 0.10	6.59 ± 0.18	4.65 ± 0.12	85.07 ± 0.29
DL	3.47 ± 0.14	6.39 ± 0.17	4.72 ± 0.18	85.43 ± 0.36
DH	3.54 ± 0.23	6.25 ± 0.36	4.55 ± 0.34	85.66 ± 0.47
WL	3.78 ± 0.19	7.04 ± 0.22	4.30 ± 0.17	84.88 ± 0.49
WH	3.42 ± 0.09	6.50 ± 0.22	3.95 ± 0.12	86.13 ± 0.20

Blood

Group	Cytotoxic T cell	T helper	Naïve CD4	Activated CD4	Naïve CD8	Activated CD8
NS	12.72 ± 1.07	20.84 ± 1.59	12.67 ± 1.20	8.83 ± 0.67	12.33 ± 1.13	1.57 ± 0.11
TC	11.67 ± 1.40	18.52 ± 2.19	11.76 ± 1.42	8.50 ± 0.90	11.16 ± 1.26	0.90 ± 0.16
DL	13.12 ± 1.14	20.64 ± 1.70	12.37 ± 1.13	8.50 ± 0.85	12.52 ± 1.13	1.29 ± 0.13
DH	11.74 ± 0.64	18.66 ± 1.02	7.01 ± 1.31	5.54 ± 1.30	10.92 ± 0.59	1.10 ± 0.08
WL	13.19 ± 0.83	20.50 ± 1.34	11.48 ± 0.76	9.43 ± 0.71	12.34 ± 0.78	1.33 ± 0.10
WH	7.86 ± 0.75 *	13.54 ± 0.80 *	7.44 ± 0.52 *	6.44 ± 0.47 *	8.03 ± 0.58 *	0.94 ± 0.10

Spleen

Group	T cells	B cells	Cytotoxic T cells	NK cells	T helper	Putative Monocytes
NS	26.58 ± 0.59	44.58 ± 0.76	20.24 ± 0.41	6.58 ± 0.55	11.69 ± 0.22	3.27 ± 0.10
TC	26.79 ± 0.54	46.13 ± 0.71	20.77 ± 0.37	6.01 ± 0.34	11.99 ± 0.23	2.94 ± 0.11
DL	27.16 ± 0.70	41.09 ± 3.08	20.70 ± 0.45	4.85 ± 0.35	12.69 ± 0.29	3.11 ± 0.15
DH	27.78 ± 0.70	43.79 ± 1.06	21.81 ± 0.46	6.38 ± 0.34	12.43 ± 0.26	3.38 ± 0.33
WL	26.32 ± 0.61	43.37 ± 0.65	19.13 ± 0.46	4.31 ± 0.24 *	11.64 ± 0.19	3.40 ± 0.14
WH	25.50 ± 0.45	43.54 ± 0.72	18.86 ± 0.46	4.69 ± 0.32 *	10.97 ± 0.29	3.15 ± 0.16

* Statistically different from control using one-way ANOVA followed by Dunnett's test for group comparisons ($P<0.05$). Groups: NS (Non-surgical); TC (Tantalum control); DL (DU-low dose); DH (DU-high dose); WL (WA-low dose); WH (WA-high dose).

TABLE 35. Flow Cytometric Analysis – 6 Month Animals

Thymus

Group	CD4-CD8-	CD4+CD8-	CD4-CD8+	CD4+CD8+
NS	6.14 ± 0.42	8.16 ± 0.34	5.01 ± 0.18	81.51 ± 0.78
TC	6.35 ± 0.20	9.23 ± 0.25	5.28 ± 0.12	79.14 ± 0.50
DL	7.31 ± 0.40	8.81 ± 0.68	5.95 ± 0.32	77.93 ± 1.02
DH	4.78 ± 0.44	6.74 ± 0.28	4.81 ± 0.24	83.67 ± 0.85
WL	4.86 ± 0.32	6.85 ± 0.19	4.67 ± 0.17	83.62 ± 0.56
WH	4.41 ± 0.22	6.75 ± 0.28	4.31 ± 0.11	84.54 ± 0.54

Blood

Group	Cytotoxic T cell	T helper	Naïve CD4	Activated CD4	Naïve CD8	Activated CD8
NS	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
TC	10.69 ± 1.23	17.18 ± 1,85	10.72 ± 1.32	7.17 ± 0.77	10.24 ± 1.21	1.23 ± 0.14
DL	9.88 ± 0.51	15.45 ± 1.07	10.66 ± 0.37	6.46 ± 1.07	9.94 ± 0.61	0.99 ± 0.09
DH	10.66 ± 1.06	16.36 ± 1.58	10.06 ± 0.92	7.03 ± 0.87	10.04 ± 1.01	1.29 ± 0.19
WL	6.07 ± 1.24 *	9.50 ± 2.27 *	5.12 ± 1.68 *	4.64 ± 1.09 *	5.45 ± 1.11 *	1.12 ± 0.56
WH	5.41± 0.89 *	8.15 ± 1.40 *	4.60 ± 0.77 *	3.76 ± 0.66 *	5.14 ± 0.93 *	0.54 ± 0.12 *

Spleen

Group	T cells	B cells	Cytotoxic T cells	NK cells	T helper	Putative Monocytes
NS	**ND**	**ND**	**ND**	**ND**	**ND**	**ND**
TC	26.69 ± 0.56	45.35 ± 0.72	20.51 ± 0.39	6.30 ± 0.44	11.84 ± 0.23	3.17 ± 0.10
DL	24.07 ± 0.44	45.63 ± 1.10	21.96 ± 0.79	9.70 ± 0.31 *	12.25 ± 0.39	4.78 ± 0.42
DH	26.65 ± 0.32	43.39 ± 1.10	21.12 ± 0.50	6.41 ± 0.56	13.39 ± 0.51	3.36 ± 0.14
WL	23.97 ± 1.00	42.76 ± 1.49	21.57 ± 1.44	9.13 ± 0.65 *	13.00 ± 0.80	4.33 ± 0.34
WH	23.69 ± 0.53*	44.64 ± 0.71	19.61 ± 0.42	10.25 ± 0.46 *	10.91 ± 0.15	3.25 ± 0.10

* Statistically different from control using one-way ANOVA followed by Dunnett's test for group comparisons ($P<0.05$). Groups: NS (Non-surgical); TC (Tantalum control); DL (DU-low dose); DH (DU-high dose); WL (WA-low dose); WH (WA-high dose). ND – not determined.

TABLE 36. Flow Cytometric Analysis – 12 Month Animals

Thymus

Group	CD4-CD8-	CD4+CD8-	CD4-CD8+	CD4+CD8+
NS	18.04 ± 2.37	14.66 ± 1.67	7.96 ± 0.43	59.34 ± 4.22
TC	21.33 ± 3.50	14.02 ± 1.27	8.68 ± 0.77	55.98 ± 5.36
DL	17.98 ± 2.07	13.47 ± 1.28	8.48 ± 0.50	60.07 ± 3.75
DH	19.38 ± 2.46	15.06 ± 1.58	8.40 ± 0.66	57.16 ± 4.63

Blood

Group	Cytotoxic T cell	T helper	Naïve CD4	Activated CD4	Naïve CD8	Activated CD8
NS	3.82 ± 0.48	6.59 ± 0.90	3.05 ± 0.53	3.82 ± 0.57	3.58 ± 0.50	0.73 ± 0.11
TC	4.53 ± 0.72	7.59 ± 1.17	3.07± 0.52	4.40 ± 0.61	3.98 ± 0.61	0.72 ± 0.14
DL	5.14 ± 1.00	8.12 ± 1.43	3.51 ± 0.69	4.97 ± 0.84	4.49 ± 0.87	0.91 ± 0.18
DH	5.90 ± 0.93	9.08 ± 1.36	4.49 ± 0.71	4.89 ± 0.72	5.26 ± 0.82	0.88 ± 0.15

Spleen

Group	T cells	B cells	Cytotoxic T cells	NK cells	T helper	Putative Monocytes
NS	23.11 ± 0.33	43.27 ± 0.79	22.34 ± 0.63	10.75 ± 0.93	10.52 ± 0.32	5.28 ± 0.61
TC	21.23 ± 0.57	40.95 ± 1.72	21.11 ± 0.31	11.47 ± 0.94	9.85 ± 0.49	7.60 ± 1.10
DL	22.11 ± 0.49	44.82 ± 0.62	21.22 ± 0.51	11.06 ± 0.65	11.12 ± 0.26	5.28 ± 0.54
DH	22.64 ± 0.44	43.21 ± 1.03	22.30 ± 0.54	11.76 ± 0.82	11.30 ± 0.35	5.61 ± 0.67

* Statistically different from control using one-way ANOVA followed by Dunnett's test for group comparisons ($P<0.05$). Groups: NS (Non-surgical); TC (Tantalum control); DL (DU-low dose); DH (DU-high dose).

Table 37. Natural Killer Cell Activity

	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High
1 Month	17.1 ± 0.9	11.4 ± 2.0	7.6 ± 1.0 *	11.6 ± 3.8	10.2 ± 1.0 *	7.5 ± 1.2 *
3 Month	19.1 ± 2.6	16.9 ± 1.4	14.3 ± 1.9	9.0 ± 2.7 *	5.8 ± 2.0 *	8.2 ± 3.0 *
6 Month	ND	ND	ND	ND	ND	ND
12 Month	ND	ND	ND	ND	ND	ND

Data expressed as the mean +/- the standard error of the mean of 15 replicates. * represents a significant difference from control at $P<0.05$ using Students t-test. ND is not detected.

Table 38. Cytotoxic T-Lymphocyte Activity

	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High
1 Month	31.2 ± 5.8	28.4 ± 5.7	21.6 ± 1.7	31.0 ± 6.6	25.1 ± 1.4	21.2 ± 1.8
3 Month	37.3 ± 2.5	20.1 ± 0.8	44.8 ± 1.9 *	44.8 ± 4.4	42.7 ± 5.5	30.0 ± 2.1 *
6 Month	37.8 ± 6.4	33.8 ± 1.0	13.5 ± 0.8 *	17.7 ± 0.7 *	20.5 ± 4.0 *	22.2 ± 3.1 *
12 Month	29.0 ± 1.8	18.2 ± 1.1	36.1 ± 4.4	34.6 ± 5.2	ND	ND

Data expressed as the mean +/- the standard error of the mean of 15 replicates. * represents a significant difference from control at $P<0.05$ using Students t-test. ND is not determined.

Table 39. Antibody Plaque-Forming Cell Activity

	Non-Surgical	Tantalum	DU-Low	DU-High	WA-Low	WA-High
1 Month	1.52 ± 0.32	3.55 ± 0.5	3.33 ± 0.35 *	1.87 ± 0.18	2.26 ± 0.55	1.84 ± 0.21
3 Month	1.23 ± 0.10	3.24 ± 0.60	1.58 ± 0.12 *	2.59 ± 0.92	0.87 ± 0.11 *	5.22 ± 0.89 *
6 Month	4.76 ± 0.62	3.02 ± 0.70	2.33 ± 0.54 *	1.16 ± 0.11 *	1.25 ± 0.29 *	ND
12 Month	3.97 ± 0.64	0.87 ± 0.20	0.74 ± 0.12 *	2.46 ± 0.35	ND	ND

Data expressed as the mean +/- the standard error of the mean of 15 replicates and represent the number of plaques (x 10e5) formed per spleen. * represents a significant difference from control
at $P<0.05$ using Students t-test. ND is not determined.

Embedded Weapons-Grade Tungsten Alloy Shrapnel Rapidly Induces Metastatic High-Grade Rhabdomyosarcomas in F344 Rats

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Continuing concern regarding the potential health and environmental effects of depleted uranium and lead has resulted in many countries adding tungsten alloy (WA)-based munitions to their battlefield arsenals as replacements for these metals. Because the alloys used in many munitions are relatively recent additions to the list of militarily relevant metals, very little is known about the health effects of these metals after internalization as embedded shrapnel. Previous work in this laboratory developed a rodent model system that mimicked shrapnel loads seen in wounded personnel from the 1991 Persian Gulf War. In the present study, we used that system and male F344 rats, implanted intramuscularly with pellets (1 mm × 2 mm cylinders) of weapons-grade WA, to simulate shrapnel wounds. Rats were implanted with 4 (low dose) or 20 pellets (high dose) of WA. Tantalum (20 pellets) and nickel (20 pellets) served as negative and positive controls, respectively. The high-dose WA-implanted rats (n = 46) developed extremely aggressive tumors surrounding the pellets within 4–5 months after implantation. The low-dose WA-implanted rats (n = 46) and nickel-implanted rats (n = 36) also developed tumors surrounding the pellets but at a slower rate. Rats implanted with tantalum (n = 46), an inert control metal, did not develop tumors. Tumor yield was 100% in both the low- and high-dose WA groups. The tumors, characterized as high-grade pleomorphic rhabdomyosarcomas by histopathology and immunohistochemical examination, rapidly metastasized to the lung and necessitated euthanasia of the animal. Significant hematologic changes, indicative of polycythemia, were also observed in the high-dose WA-implanted rats. These changes were apparent as early as 1 month postimplantation in the high-dose WA rats, well before any overt signs of tumor development. These results point out the need for further studies investigating the health effects of tungsten and tungsten-based alloys.

Key words: **cobalt, embedded fragment, nickel, rat, rhabdomyosarcoma, tungsten, tungsten alloy.** ***Environ Health Perspect*** **113:729–734 (2005). doi:10.1289/ehp.7791 available via *http://dx.doi.org/* [Online 15 February 2005]**

Tungsten has been used for many years in a variety of applications. Combining the hard, brittle tungsten metal with various other metals, including nickel and cobalt, produces tungsten alloys (WAs) with specific characteristics, some of which are of interest to the military. Recently, WAs have replaced lead in some small-caliber ammunition (the "green bullet") [Oak Ridge National Laboratory (ORNL) 1998] and depleted uranium (DU) in kinetic-energy penetrators (ORNL 1996). Based on a small number of studies, prevailing theory is that elemental tungsten or insoluble tungsten compounds have only limited toxicity (Leggett 1997). For example, tungsten coils implanted into the subclavian artery of rabbits rapidly degrade, leading to elevated serum tungsten levels as early as 15 min after implantation. However, after 4 months, no signs of local or systemic toxicity were observed (Peuster et al. 2003). Studies on health effects of Ni and Co are more numerous. Intramuscular injections (28 mg) of soluble metallic Ni or Co result in formation of rhabdomyosarcomas at the injection site. With Ni, 100% of injected rats develop a tumor within 41 weeks (Heath and Daniel 1964), whereas administration of Co results in tumor formation in 40% of the rats with a latency period of 71 weeks (Heath 1954, 1956). However, intramuscular implantation of rods or pellets composed of various Ni or Co alloys used in orthopedic prosthetics results in no excessive tumor formation (Gaechter et al. 1977; Sunderman 1989). A variety of other Ni compounds, including nickel subsulfide, nickel oxide, and nickel monosulfide, have been tested for carcinogenic potential via intramuscular administration (Gilman 1962; Sunderman and Maenza 1976; Sunderman et al. 1977). Tumors (rhabdomyosarcoma and fibrosarcoma) were found in many cases at the injection site, with tumor yield dependent on solubility and concentration of the administered compound. It has been postulated that the yield of localized tumors is inversely related to the rate of solubilization of the Ni-containing compound (Kasprzak et al. 1983). This hypothesis does not appear to hold for Co compounds (Lison et al. 2001).

Metal alloys present additional problems when investigating health effects. The various metals comprising the alloy, as well as the method of production, can all factor into the overall health effect observed upon exposure. Investigations on hard-metal disease have shown that either tungsten carbide or Co alone has limited toxicity on lung tissue (Lasfargues et al. 1992). However, when combined, the tungsten carbide/cobalt mixture acts synergistically to increase the observed toxicity. It is not known whether this is due to the combined toxicity of the tungsten carbide/cobalt mixture or to an increase in the bioavailability of the known toxicant, Co (Lison and Lauwerys 1997). *In vitro* studies investigating malignant transformation of immortalized human cells by mixtures of tungsten, Ni, and Co suggest a synergistic effect that greatly exceeds the effects of the metals individually (Miller et al. 2001, 2002).

Advancements in metallurgy have led the military of many nations to replace DU in some armor-penetrating munitions and lead in small-caliber ammunition with various alloys of tungsten. One motivation for such a replacement is widespread public concern about the health and environmental impact of continued use of these metals. However, to our knowledge, none of these militarily relevant WAs has been tested for potential health effects, especially as embedded shrapnel. There is a growing list of health concerns related to tungsten exposure. Although a definitive link has not been established, several cancer clusters in the United States are associated with elevated levels of tungsten in the environment. Those findings, along with the results presented in this article, raise questions about the possible consequences of tungsten exposure. More important, these results raise extremely serious concerns over the potential health effects of WA-based munitions currently being used as nontoxic alternatives to lead and DU.

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Materials and Methods

Rodents. Male F344 rats (6 weeks of age; Harlan, Frederick, MD) were maintained in a facility accredited by the Association of Assessment and Accreditation of Laboratory Animal Care in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources 1996). All procedures, including euthanasia criteria (Tomasovic et al. 1988), were approved by the Armed Forces Radiobiology Research Institute's (AFRRI) Animal Care and Use Committee. Upon arrival, animals were screened for common rodent pathogens. Rats were pair-housed in plastic microisolator cages with hardwood chips for bedding and fed a certified NTP-2000 (Quality Lab Products, Elkridge, MD) diet (Rao 1996) with acidified water provided *ad libitum*. Animals were on a 12-hr light/dark cycle with no twilight and were weighed weekly.

Pellets. All metal pellets were cylinders 1 mm in diameter and 2 mm in length. Nickel (99.995% metallic Ni) and tantalum (99.95% Ta) pellets were purchased from Alfa Aesar (Ward Hill, MA). WA pellets were fabricated by Aerojet Ordnance Tennessee (Jonesborough, TN) using standard kinetic energy penetrator production processes. An average WA pellet weighed 27.5 mg and consisted of 91.1% tungsten, 6.0% Ni, and 2.9% Co. Ni and Ta pellets weighed 14 mg and 27 mg, respectively. Before implantation surgery, all pellets were cleaned and chemically sterilized (Pellmar et al. 1999).

Pellet-implantation surgery. A rodent model system (AFRRI 1996), originally developed to mimic DU shrapnel loads seen in wounded personnel from the 1991 Persian Gulf War, was used to investigate the health effects of retained WA shrapnel. All rats were implanted with a total of 20 pellets split evenly between each hind leg. Experimental groups included Ta (negative control, 20 Ta pellets), low-dose WA (4 WA pellets and 16 Ta pellets), high-dose WA (20 WA pellets), and Ni (positive control, 20 Ni pellets). Tantalum was used as a negative implantation control because it is considered inert and has been used in human prostheses (Hockley et al. 1990; Johansson et al. 1990). Nickel, a known carcinogen, was used as a positive control (Costa and Klein 1999; Kasprzak et al. 2003). Rats were implanted at 9 weeks of age. For the pellet implantation procedure, anesthesia was induced by continuous administration of isoflurane using an open circuit system with a scavenger/recapture system. All surgery was done using aseptic techniques. After the surgical sites were clipped and cleansed with Betadine, an incision was made through the skin to expose the gastrocnemius muscle. Pellets were implanted in the muscle, spaced approximately 1.5 mm apart on the lateral side of each leg. The incision was closed with sutures and tissue adhesive. Rats were closely monitored after surgery until they were ambulatory. An analgesic (buprenorphine hydrochloride; Reckitt and Colman, Hull, UK) was administered preoperatively and then as needed postoperatively. The surgical sites were examined daily for signs of inflammation, infection, and local metal toxicity.

Experimental groups. Our pellet implantation groups included Ta (negative control), WA (both a low- and high-dose group), and Ni (positive control). The original euthanasia time points were to be 1, 3, 6, 12, 18, and 24 months; however, because of the rapid tumor development, no WA- or Ni-implanted rat survived much past 6 months postimplantation. Final survival data therefore included rats originally assigned to the 12-, 18-, and 24-month experimental groups, whose animals died earlier than those designated time points. This resulted in group sizes of $n = 46$ for the Ta and both WA groups, and $n = 36$ for the Ni group. Hematologic assessments were conducted on the separate 1-, 3-, and 6-month WA implantation groups.

Pathology. At various times postimplantation or when moribund, rats were euthanized by isoflurane overdose. A complete gross pathology examination was conducted, noting any abnormalities, and tissues were collected for analysis. Weights of representative tissues, including spleen, thymus, testes, kidney, and liver, were determined and normalized to body weight. Tissues for histopathology were fixed in buffered formalin, processed and embedded in paraffin, cut at 5–6 µm, mounted, and stained with hematoxylin and eosin (H&E). Immunohistochemical analysis was conducted on 5-µm-thick sections of formalin-fixed, paraffinized tissue. After deparaffination and rehydration, nonspecific binding was blocked with Power Block (Biogenex, San

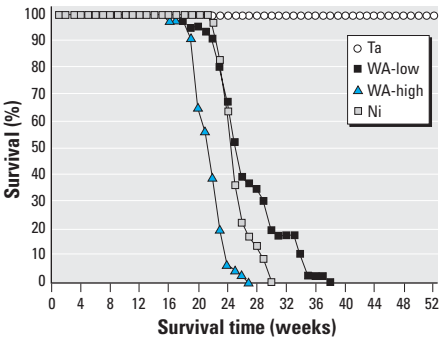

Figure 1. Survival times of pellet-implanted rats.

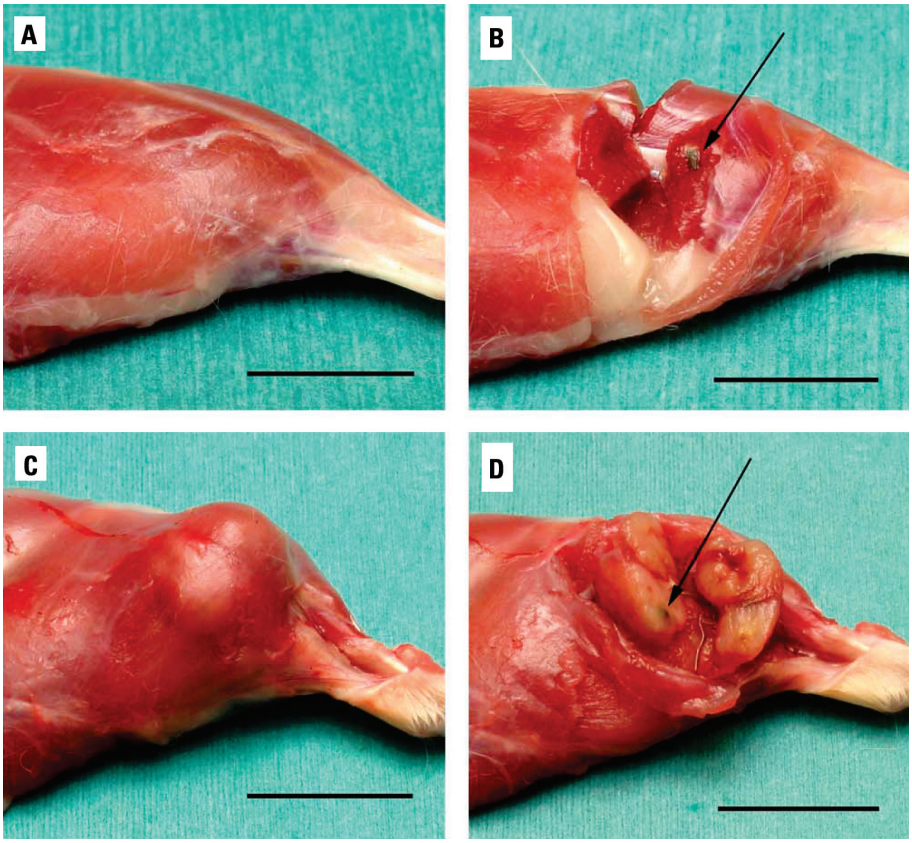

Figure 2. Effect of implanted WA pellets on F344 rats. (*A*) Gross appearance of Ta-implanted hind leg. (*B*) Dissected area around implanted Ta pellet (arrow indicates pellet). (*C*) Gross appearance of WA-implanted hind leg with tumor(s). (*D*) Dissected area around implanted WA pellet with tumor surrounding pellet (arrow indicates pellet). Bar = 2 cm.

Ramon, CA). The tissue was then reacted with prediluted rabbit anti-desmin polyclonal antibody (Biogenex) and treated with biotinylated secondary anti-rabbit antibody (Biogenex). After blocking with hydrogen peroxide, the tissue sections were labeled with peroxidase-conjugated streptavidin (Biogenex) and aminoethyl carbazole (AEC; Biogenex) was used as a chromogen. Slides were then counterstained with hematoxylin and mounted.

Hematology. At euthanasia, we obtained blood for hematologic assessments from the abdominal aorta of isoflurane-anesthetized rats using a heparinized needle and sample tubes containing EDTA (Becton-Dickinson, Franklin Lakes, NJ). We determined white and red blood cell counts; hemoglobin; hematocrit; mean corpuscular volume, hemoglobin, and hemoglobin concentration; red cell distribution width; platelet counts and volume; and neutrophil, lymphocyte, monocyte, eosinophil, and basophil counts with a Bayer Advia 120 Hematology Analyzer (Bayer Diagnostics, Terrytown, NY).

Results

All rats tolerated the pellet implantation procedure with no apparent adverse effects. The incision sites were examined daily; no rat showed any signs of infection from the surgery, or any discomfort postoperatively. Body weights were recorded weekly. Once they had recovered from the surgical procedure, all rats gained weight at equivalent rates. However, in the first week after the pellet implantation surgery, the rate of weight gain by the Ta and low-dose WA rats was slower than normal, and high-dose WA and Ni rats lost weight. This was followed by large weight gains in postimplantation week 2 in all experimental groups. There were no statistical differences in rate of body weight gain between any of the groups throughout the remaining experimental period. As previously reported, the implantation and retention of cylindrical metal pellets (1 mm × 2 mm) had no effect on locomotive abilities in rats (AFRRI 1996; Pellmar et al. 1999), nor did we observe any such difficulties in this study.

At approximately 16–20 weeks postimplantation, we began to observe tumors at the pellet implantation sites in the WA and Ni rats. In some high-dose WA animals, palpable tumors were apparent as early as 14 weeks postimplantation. Tumors developed rapidly in WA-implanted animals. The tumors were aggressive and fast growing, necessitating euthanasia of the animals several weeks later. On the basis of previously published literature (Heath and Daniel 1964), we expected the Ni-implanted positive control rats to develop tumors at the implantation site, but the speed at which the tumors developed was surprising: approximately 5 months after implantation.

Figure 1 shows the percentage of surviving animals as a function of time after pellet implantation. Rats implanted with Ta pellets (n = 46) survived well beyond 12 months with no apparent health problems. All rats in the high- and low-WA and the Ni groups developed tumors and were euthanized upon becoming moribund. Rats in the high-dose WA group (n = 46) survived the least amount of time (mean survival time ± SD = 21.8 ± 2.1 weeks). Nickel-implanted animals (n = 36) and the low-dose WA group (n = 46) survived slightly longer, with mean (± SD) survival times of 25.4 ± 2.1 and 27.0 ± 4.6 weeks, respectively. The mean survival time of the high-dose WA animals was significantly shorter than that of the low-dose WA- or Ni-implanted animals [analysis of variance (ANOVA) followed by Dunnett's test, $p < 0.05$]. The mean survival times of the low-dose WA- and the Ni-implanted animals were not statistically different from each other. The

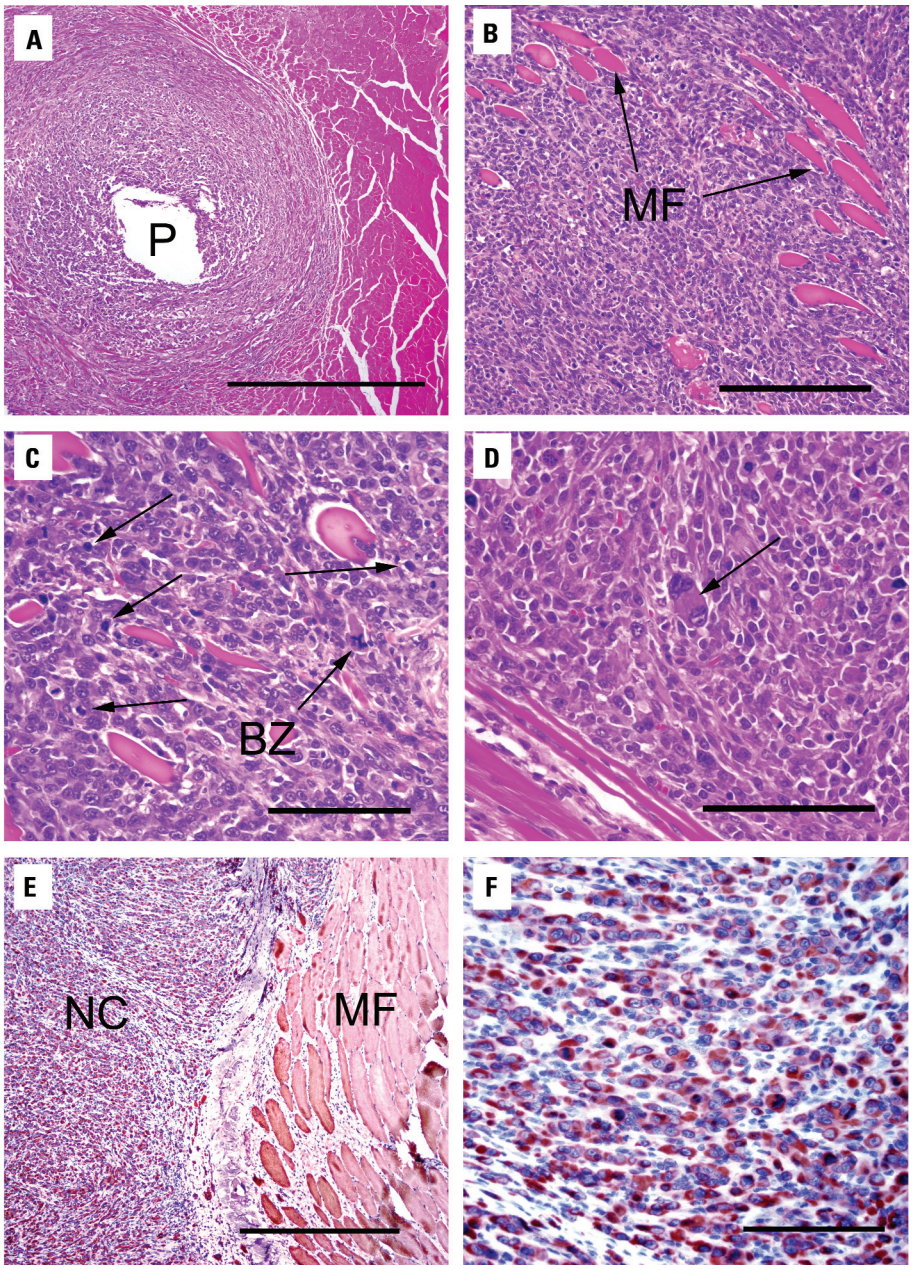

Figure 3. Histopathologic examination of leg tumor surrounding WA pellet. (*A*) H&E-stained section of leg tumor from F344 rat showing WA pellet hole (P); bar = 500 µm. (*B*) H&E-stained tumor section showing neoplastic infiltration of preexisting muscle fibers (MF); bar = 200 µm. (*C*) H&E-stained tumor section showing neoplastic cells with numerous mitoses (arrows) and bizarre mitotic figures (BZ); bar = 100 µm. (*D*) H&E-stained tumor section showing pleomorphic cell (arrow); bar = 100 µm. (*E*) Desmin staining of leg tumor showing neoplastic cells (NC) and muscle fibers (MF); bar = 500 µm. (*F*) Desmin staining of neoplastic cells; bar = 50 µm.

results reported here are part of a larger study that also investigated the health effect of embedded DU fragments. We did not observe tumor formation in the DU-implanted rats (Kalinich JF, Miller AC, McClain DE, unpublished data).

Upon euthanasia, the animals underwent necropsy, and tissue samples were taken for various analyses. Figure 2 shows the appearance of the hind limb of rats implanted with Ta (Figure 2A) or WA (Figure 2C) for 26 and 23 weeks, respectively, before surgical removal of the implanted pellets. The gross anatomy of the Ta-implanted leg is normal, whereas in the WA leg the tumor is clearly visible. Upon dissection, no obvious abnormalities were observed in the Ta-implanted animals, and the pellets could be easily removed (Figure 2B). However, in the WA-implanted animals, the pellets were surrounded by tumor (Figure 2D). In many cases, the interior of the tumor had become necrotic and/or hemorrhagic. Similar tumors were found for both WA- and Ni-implanted animals. In low-dose WA animals, tumors were found surrounding the WA pellets only. No tumors were found surrounding implanted Ta pellets. Implanted WA pellets rapidly oxidized and had a slightly eroded appearance. Ta pellets did not have an eroded appearance even after implantation for 6 months. However, despite their appearance, the WA pellets lost < 5% of their mass over this time.

Tumor tissue was histopathologically examined and characterized. Figure 3A shows the neoplastic cells surrounding the site of the implanted WA pellet. These cells infiltrated preexisting skeletal muscle fibers. Fibers that became isolated by this process degenerated and demonstrated a loss of cross-striations and internalization of nuclei (Figure 3B,C). Neoplastic cells were pleomorphic with marked anisocytosis and anisokaryosis (Figure 3D). In addition, an extremely high mitotic rate was observed in these cells, and bizarre mitoses were present. Immunohistochemical staining was used to determine the origin of these neoplastic cells. The cells were strongly positive for desmin (Figure 3E,F), suggesting a skeletal muscle origin.

In the WA-implanted animals, the tumors had metastasized to the lung. None of the Ni-implanted animals showed signs of lung metastases, although some exhibited endogenous histiocytic lipid pneumonia not seen in the WA animals. Figure 4A shows numerous metastatic foci in the lungs of a high-dose WA rat. These multiple masses obscure > 50% of the lung surface and up to 90% in the latter stages of development. Figure 4B shows a photomicrograph of these pulmonary metastases. Apparent is the multifocal, vascular orientation of these neoplasms. There are neoplastic cells surrounding the arterioles and bronchioles, expanding the alveolar septae, and replacing alveolar spaces. These neoplastic cells have a high mitotic rate and are often seen surrounding or occluding arterioles (Figure 4C). Figure 4D shows that the metastatic neoplastic cells, as well as vascular and airway smooth muscle, are strongly positive for the muscle marker desmin.

Selected hematologic and organ weight parameters for euthanized rats are shown in Table 1. The Ta data were obtained from rats implanted with Ta pellets for 6 months. The data for the remaining groups were obtained at the time the rats became moribund because of tumor development. No significant differences in organ/body weight ratios were seen for the low-dose WA- or Ni-implanted animals compared with Ta-implanted control rats. However, high-dose WA-implanted rats showed significantly higher spleen:body weight ratios compared with control rats. In addition, thymus:body weight ratios were decreased in the high-dose WA rats. Because the spleen and thymus are integral components of the immune system, these changes suggest that embedded WA, at certain levels, may be immunotoxic. The kidney:body weight ratio for high-dose WA rats was also significantly higher than that of Ta-implanted rats. High-dose WA rats euthanized 1 and 3 months after pellet implantation also exhibited significantly elevated spleen:body weight ratios compared with the appropriate Ta-implanted control rats (Tables 2 and 3). Thymus:body weight ratios, however, were not significantly different. At 3 months postimplantation, the kidney:body weight ratio in high-dose WA rats was significantly higher than that in Ta rats, but it was significantly lower at 1 month postimplantation. There were no 1- and 3-month Ni-implanted groups.

WA-implanted animals had significant changes in a number of hematologic parameters. Rats implanted with 20 WA pellets exhibited significant increases in white blood cell counts, red blood cell counts, hemoglobin, and hematocrit levels compared with Ta control rats, whereas rats implanted with 20 Ni pellets had significant decreases in red blood cell counts, hemoglobin, and hematocrit levels (Table 1). Hematologic parameters from low-dose WA rats were not statistically different from controls. Statistically significant increases in red blood counts, hemoglobin, and hematocrit levels were observed in high-dose WA animals as early as 1 month after pellet implantation and persisted throughout

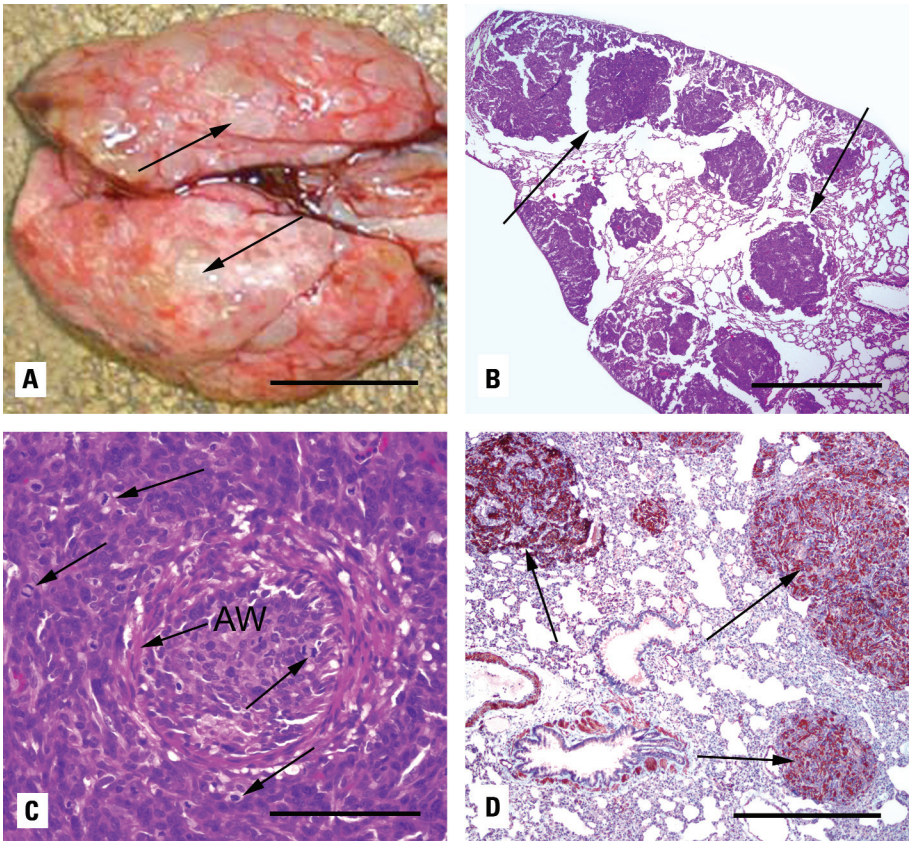

Figure 4. Lung metastases from WA-implanted F344 rats. (*A*) Gross appearance of pulmonary metastases from WA-implanted rat (arrows indicate metastatic foci); bar = 1 cm. (*B*) H&E-stained section of pulmonary metastases (arrows); bar = 1 mm. (*C*) H&E-stained section of an occluded pulmonary arteriole [arrow indicates vascular smooth muscle wall (AW)] showing neoplastic cells with numerous mitoses (arrows); bar = 50 µm. (*D*) Desmin staining of pulmonary metastases (arrows); bar = 500 µm.

the experimental period (Tables 2 and 3). In addition, there were statistically significant increases in the numbers of neutrophils, lymphocytes, monocytes, and eosinophils present in high-dose WA animals. Low-dose WA animals had elevated neutrophil, lymphocyte, and monocyte numbers at 3 months postimplantation, but only the neutrophil numbers were statistically different from the controls at the 5–6 month euthanasia point. The Ni-implanted animals had significantly lower lymphocyte counts than the controls. All other parameters were statistically identical to the controls. These results suggest there is a dose-dependent perturbation in many hematology parameters as a result of an increasing WA pellet number.

Discussion

Tungsten-based alloys are currently being used as replacements for DU in kinetic-energy penetrators and for lead in small-caliber ammunition. However, the health effects of these unique alloys have not been investigated, especially in the case of embedded fragments such as shrapnel wounds. In this study, using male F344 rats and a system designed to investigate the effects of embedded metal fragments (AFRRI 1996), we have shown the embedded weapons-grade WA (91.1% W, 6.0% Ni, 2.9% Co) results in rapid tumor formation at the implantation site in 100% of the rats. The rate of tumor formation correlates with pellet number. Ni-implanted rats also develop tumors at the implantation site, although not as rapidly as seen with WA. Histopathologic and immunohistochemical data support a diagnosis of a pleomorphic rhabdomyosarcoma for both the WA- and Ni-induced leg tumors (Altmannsberger et al. 1985).

Rats implanted with 20 WA pellets (high-dose WA) showed significantly increased spleen:body weight ratios compared with Ta control rats. Low-dose WA rats (four WA pellets) also exhibited increased spleen:body weight ratios, but these increases were not statistically significant (ANOVA followed by Dunnett's test). Values for Ni-implanted rats were identical to control rats. The spleen changes observed in the high-dose WA rats were apparent as early as 1 month after pellet implantation. Once again, low-dose WA rats showed increased, but not statistically significant, spleen:body weight ratios. With the exception of the spleen, the only other organ:body weight perturbations were seen in high-dose WA rats and included a decrease in thymus:body weight ratio at approximately 5 months and changes in kidney:body weight ratios. The 1-month kidney:body weight ratio for high-dose WA rats was significantly lower

Table 1. Selected hematologic and organ weight parameters (mean ± SEM) for euthanized rats.

	Ta	WA (low)	WA (high)	Ni
White blood cells (10^3/μL)	3.19 ± 0.24	3.95 ± 0.43	4.56 ± 0.29*	2.56 ± 0.20
Red blood cells (10^6/μL)	8.32 ± 0.09	8.03 ± 0.19	10.10 ± 0.07**	7.46 ± 0.13**
Hemoglobin (g/dL)	14.50 ± 0.13	13.90 ± 0.36	16.46 ± 0.30**	12.95 ± 0.23**
Hematocrit (%)	41.77 ± 0.53	40.38 ± 0.96	50.18 ± 0.39**	38.12 ± 0.77**
MCV (fL)	50.22 ± 0.16	50.26 ± 0.28	49.71 ± 0.16	51.08 ± 0.66
MCH (pg)	17.46 ± 0.15	17.31 ± 0.13	16.30 ± 0.28**	17.35 ± 0.08
MCHC (g/dL)	34.77 ± 0.36	34.46 ± 0.32	32.81 ± 0.62**	34.05 ± 0.50
RDW (%)	12.54 ± 0.09	13.07 ± 0.11**	13.77 ± 0.09**	13.04 ± 0.16*
Platelets (10^3/μL)	562.00 ± 14.72	542.05 ± 14.27	467.50 ± 17.57**	487.18 ± 26.10*
MPV (fL)	9.93 ± 0.69	8.64 ± 0.52	10.13 ± 0.62	8.97 ± 0.52
Neutrophils (10^3/μL)	0.79 ± 0.05	1.03 ± 0.09*	1.31 ± 0.12**	0.78 ± 0.09
Lymphocytes (10^3/μL)	2.21 ± 0.18	2.42 ± 0.17	2.95 ± 0.23*	1.63 ± 0.12*
Monocytes (10^3/μL)	0.07 ± 0.01	0.09 ± 0.02	0.13 ± 0.02*	0.05 ± 0.01
Eosinophils (10^3/μL)	0.08 ± 0.01	0.08 ± 0.01	0.12 ± 0.01**	0.06 ± 0.01
Basophils (10^3/μL)	0.02 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.02 ± 0.00
Spleen (mg/g bw)	2.18 ± 0.10	2.30 ± 0.08	2.60 ± 0.06**	2.17 ± 0.05
Thymus (mg/g bw)	0.86 ± 0.03	0.76 ± 0.04	0.70 ± 0.04*	0.74 ± 0.07
Liver (mg/g bw)	29.21 ± 0.28	29.39 ± 0.24	28.77 ± 0.35	29.52 ± 0.39
Kidney (mg/g bw)	5.13 ± 0.06	5.13 ± 0.06	5.36 ± 0.05*	5.15 ± 0.08
Testes (mg/g bw)	7.31 ± 0.07	7.20 ± 0.08	7.40 ± 0.10	7.21 ± 0.14

Abbreviations: bw, body weight; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; RDW, red blood cell distribution width. Data represent mean ± SEM of 20 observations (10 for Ni group).
*$p < 0.05$, and **$p < 0.01$ compared with the Ta control group by one-way ANOVA followed by Dunnett's test for group mean comparisons.

Table 2. Selected hematologic and organ weight parameters (mean ± SEM) for rats implanted with metal pellets for 3 months.

	Ta	WA (low)	WA (high)
White blood cells (10^3/μL)	2.88 ± 0.20	4.06 ± 0.14**	4.01 ± 0.21**
Red blood cells (10^6/μL)	7.48 ± 0.06	8.48 ± 0.15*	9.10 ± 0.70**
Hemoglobin (g/dL)	12.90 ± 0.09	15.48 ± 0.35*	17.29 ± 0.15**
Hematocrit (%)	38.10 ± 0.27	42.14 ± 0.73*	44.79 ± 0.62**
MCV (fL)	50.96 ± 0.45	49.70 ± 0.09	48.87 ± 0.39
MCH (pg)	17.26 ± 0.12	18.27 ± 0.17	17.65 ± 0.12
MCHC (g/dL)	33.84 ± 0.35	36.71 ± 0.31**	35.89 ± 0.31**
RDW (%)	12.82 ± 0.33	12.68 ± 0.12	13.61 ± 0.09**
Platelets (10^3/μL)	513.20 ± 38.36	585.11 ± 35.87	568.29 ± 8.82
MPV (fL)	9.58 ± 1.13	9.14 ± 0.59	11.74 ± 0.51
Neutrophils (10^3/μL)	0.62 ± 0.04	0.79 ± 0.03*	0.91 ± 0.08*
Lymphocytes (10^3/μL)	2.10 ± 0.16	3.06 ± 0.14*	2.82 ± 0.17*
Monocytes (10^3/μL)	0.04 ± 0.01	0.07 ± 0.01*	0.08 ± 0.01*
Eosinophils (10^3/μL)	0.09 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
Basophils (10^3/μL)	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.00
Spleen (mg/g bw)	2.07 ± 0.03	2.16 ± 0.03	2.50 ± 0.03**
Thymus (mg/g bw)	0.73 ± 0.03	0.84 ± 0.03	0.70 ± 0.04
Liver (mg/g bw)	30.58 ± 0.33	31.00 ± 0.33	30.27 ± 0.31
Kidney (mg/g bw)	5.43 ± 0.06	5.73 ± 0.23	5.76 ± 0.04**
Testes (mg/g bw)	8.34 ± 0.12	8.21 ± 0.46	8.42 ± 0.18

Abbreviations: bw, body weight; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; RDW, red blood cell distribution width. Data represent mean ± SEM of 15 observations.
*$p < 0.05$, and **$p < 0.01$ compared with the age-matched Ta control group by one-way ANOVA followed by Dunnett's test for group mean comparisons.

Table 3. Selected hematologic and organ weight parameters (mean ± SEM) for rats implanted with metal pellets for 1 month.

	Ta	WA (low)	WA (high)
White blood cells (10^3/μL)	3.86 ± 0.20	3.81 ± 0.14	3.86 ± 0.21
Red blood cells (10^6/μL)	7.84 ± 0.08	7.74 ± 0.07	8.50 ± 0.07**
Hemoglobin (g/dL)	13.65 ± 0.15	14.81 ± 0.16	15.84 ± 0.14**
Hematocrit (%)	40.15 ± 0.42	39.66 ± 0.50	43.29 ± 0.35**
MCV (fL)	51.20 ± 0.14	51.22 ± 0.31	50.98 ± 0.19
MCH (pg)	17.41 ± 0.05	19.12 ± 0.09	18.64 ± 0.19**
MCHC (g/dL)	34.01 ± 0.12	37.37 ± 0.29	36.56 ± 0.41**
RDW (%)	12.21 ± 0.11	12.69 ± 0.11	14.18 ± 0.18**
Platelets (10^3/μL)	646.50 ± 18.76	641.00 ± 17.97	756.20 ± 43.48*
MPV (fL)	7.91 ± 0.40	8.56 ± 0.39	9.90 ± 0.55*
Neutrophils (10^3/μL)	0.65 ± 0.04	0.79 ± 0.05	0.81 ± 0.04**
Lymphocytes (10^3/μL)	3.04 ± 0.18	2.85 ± 0.13	2.90 ± 0.18
Monocytes (10^3/μL)	0.06 ± 0.00	0.06 ± 0.01	0.07 ± 0.00
Eosinophils (10^3/μL)	0.07 ± 0.01	0.08 ± 0.01	0.05 ± 0.00*
Basophils (10^3/μL)	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00
Spleen (mg/g bw)	2.37 ± 0.06	2.42 ± 0.05	2.73 ± 0.04**
Thymus (mg/g bw)	1.07 ± 0.03	1.14 ± 0.04	1.06 ± 0.03
Liver ((mg/g bw)	34.47 ± 0.26	34.31 ± 0.22	34.18 ± 0.61
Kidney (mg/g bw)	6.17 ± 0.08	6.06 ± 0.06	5.91 ± 0.05*
Testes (mg/g bw)	10.10 ± 0.16	9.86 ± 0.13	9.98 ± 0.11

Abbreviations: bw, body weight; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MPV, mean platelet volume; RDW, red blood cell distribution width. Data represent mean ± SEM of 15 observations.
*$p < 0.05$, and **$p < 0.01$ compared with the age-matched Ta control group by one-way ANOVA followed by Dunnett's test for group mean comparisons.

than control. However, from 3 months on, these ratios were significantly higher than control. It is possible that the lower kidney weights at 1 month postimplantation represent a toxic response to the heavy metals from the implanted pellets, but by 3 months and later, the kidney has begun to respond in a different manner. Although there were no gross abnormalities of the kidney at necropsy, we continue to investigate this observation.

A variety of hematologic changes were observed in WA- and Ni-implanted rats. Ni-implanted rats showed a significant decrease in red blood cells, hemoglobin, and hematocrit at the time of morbidity, indicating possible Ni-induced anemia. For low-dose WA rats the hematologic changes, including significant increases in red blood cells, white blood cells, hemoglobin, hematocrit, neutrophils, lymphocytes, and monocytes, peaked at 3 months postimplantation and returned to normal by 5–6 months. High-dose WA rats demonstrated the same changes observed in low-dose WA rats, but they occurred much more rapidly (as early as 1 month postimplantation) and persisted throughout the life of the animal. The splenomegaly and hematologic changes observed in these rats are suggestive of polycythemia. Cobalt has been used experimentally to induce polycythemia in rats (Endoh et al. 2000; Rakusan et al. 2001), although the concentration required is far greater than found in the WA pellets. In addition, the speed at which these hematologic changes occurred in the high-dose WA rats was also surprising. These results suggest a dose-dependent perturbation in many hematology parameters as a result of an increasing WA pellet number.

The search for munitions that are considered environmentally friendly yet still retain their military effectiveness has led to the appearance of many unique alloys on the modern battlefield. Often, decisions on the health consequences of exposure (inhalation, ingestion, wound contamination, etc.) to these specific alloys are based on studies that investigated only one specific metal of the alloy rather than the particular alloy in question. Tungsten-based munitions are a recent addition to many countries' arsenals, primarily in response to the continuing concerns regarding the potential environmental and health effects of DU in kinetic-energy penetrators and of lead in small-caliber ammunition. For years, exposure to tungsten was thought to be of little consequence to health. In fact, tungsten is occasionally found as a minor component in some of the various alloys used to produce medical implant devices such as artificial hips and knees. The tungsten concentration in these alloys ranges from 5% to 15%. Because the alloy used in WA munitions usually contains > 90% tungsten, along with smaller amounts of other metals, it was also assumed that exposure to these alloys would present little or no health risk. As we have shown here, this is not the case in our rodent model. Embedded WA pellets not only resulted in aggressive, metastatic, pleomorphic rhabdomyosarcomas, but also caused significant hematopoietic changes well before the carcinogenic effect was observed. It seems unlikely that these adverse health effects can be attributed solely to the small amounts of Ni and/or Co present in the alloy. The tumors induced by the 100% Ni implants occurred later than those induced by the alloys containing 6% Ni. However, recent *in vitro* studies have demonstrated a synergistic effect in terms of damage when tungsten is present with these metals (Miller et al. 2001, 2002).

The mechanism of the effects reported here with embedded WA pellets remains unclear. Despite the fact that the smooth and impermeable surface of the pellets represent characteristics known capable of inducing foreign-body or solid-state carcinogenesis (Bates and Klein 1966; Brand et al. 1975), this process is unlikely to have occurred in our experiments because implanted Ta pellets of an identical geometry and surface resulted in no tumor formation. One possibility is that free-radical reactions at the interface of the pellet and tissue could result in damage leading to carcinogenesis. Recently, the role of tungsten in human health and disease has come under increased scrutiny. Environmental testing of the leukemia cluster around Fallon, Nevada, in the United States showed slightly elevated levels of several heavy metals including uranium and Co but significantly elevated levels of tungsten [Centers for Disease Control and Prevention (CDC) 2003]. Although no definitive link between elevated tungsten levels and cancer has been established, because of the uncertainty surrounding this issue, the U.S. National Toxicology Program recently added tungsten to their list of compounds to be assessed for adverse health effects. Further study of the health effect of tungsten and WAs is clearly indicated.

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Status of Health Concerns about Military Use of Depleted Uranium and Surrogate Metals in Armor-Penetrating Munitions

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ABSTRACT

The use of depleted uranium in armor-penetrating munitions remains a source of controversy because of the numerous unanswered questions about its long-term health effects. Although there are no conclusive epidemiological data correlating depleted uranium exposure to specific health effects, studies using cultured cells and laboratory rodents continue to suggest the possibility of genetic, reproductive, and neurological effects from chronic exposure. Until issues of concern are resolved with further research, the use of depleted uranium by the military will continue to be controversial. Meanwhile, there are military programs to find substitutes for depleted uranium in munitions. Although a wide variety of alloys are being evaluated by munitions developers, certain alloys of tungsten have been developed that demonstrate properties very close to the ones that make depleted uranium useful in armor-penetrating munitions. One hundred and fifty years of industrial experience suggest that tungsten and tungsten alloys are not a significant health risk except in certain industrial exposure scenarios. However, recent research has shown that some of the most promising militarily relevant alloys of tungsten exhibit unexpected long-term toxicities as embedded shrapnel. Rats implanted in their leg muscles with pellets made from a particular alloy of tungsten, nickel, and cobalt, considered a promising surrogate for depleted uranium in munitions, develop aggressive rhabdomyosarcomas within 6 months of implantation that metastasize to the lung and necessitate euthanasia of the animals. One hundred percent of the tungsten alloy-implanted rats were affected. Immune system changes independent of tumor development were also observed. These findings amplify the need to investigate substances of questionable toxicity early in munitions development, especially with regards to the unusual kinds and levels of exposure that might be expected by the military.

1.0 INTRODUCTION

Advances in metallurgy and weapons design in the past several decades have led to new munitions whose effectiveness has provided tactical advantages on the battlefield and consequently, saved lives of personnel. However, decisions to deploy these munitions have sometimes outpaced our knowledge of how they impact the health of those exposed to them.

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Depleted uranium (DU) kinetic energy penetrators are perhaps the best-known example of these advanced munitions, primarily because of their outstanding, well-publicized performance against enemy armor in the 1991 Persian Gulf War. DU munitions were again used in the NATO military actions in Bosnia-Herzegovina (1995) and Kosovo (1999) and more recently, the U.S. led invasions of Afghanistan and Iraq.

Depleted uranium (DU) munitions were used only by Coalition forces during the 1991 Gulf War, but their use led to DU fragment injuries among Coalition forces as a result of friendly fire incidents. Other personnel were exposed via inhalation/ingestion after working around vehicles struck by DU munitions. Such exposures were not considered especially dangerous at the time, because numerous epidemiological studies of uranium miners and millers working with natural uranium had shown few concrete health effects from exposure; and DU has 40% less radioactivity than natural uranium. However, the exposure of wounded personnel to uranium as embedded fragments had no medical precedent, so the earlier studies dealing primarily with inhalation or ingestion exposures in miners were of uncertain utility. As a result, questions were soon raised as to whether it was wise to leave in place fragments possessing the unique radiological and toxicological properties of DU, especially when considering that exposures might extend as long as the 40-50 years remaining in the individuals' lives. As these treatment questions were being addressed, a growing public concern about the long-term health and environmental impact of using a radioactive metal like DU on the battlefield fueled forceful national and international efforts to ban the use of DU in munitions.

The medical and political controversies surrounding the use of DU played an important part in stimulating a search for substitute metals in armor-penetrating munitions. A wide variety of alloys have been and are being investigated, but recent developments in tungsten metallurgy have led to new alloys of tungsten/nickel/cobalt and tungsten/nickel/iron that rival DU in armor-penetrating performance. They have become among the leading candidates to replace DU in selected munitions, and ordnance containing these alloys has already been deployed, although on a relatively small scale.

This report aims to summarize the current status of knowledge about the health effects of DU and the general class of tungsten alloys currently being considered as surrogates for DU in munitions. DU toxicity at moderate exposure levels appears to be low in most cases, but significant questions have been raised about the toxicity of the tungsten alloys. Our present understanding and experience reinforces the advisability of including more effective health effects testing early in weapons development programs. The relatively insignificant cost of such testing would be paid back many times over by helping to redirect expensive engineering programs to more acceptable alternatives earlier in the development process.

2.0 DEPLETED URANIUM

2.1 Background

Uranium was discovered in the mineral pitchblende in 1789 by the German chemist Martin Heinrich Klaproth. Uranium does not exist in pure metallic form in nature because it is quickly oxidized in air. It occurs most commonly as U_3O_8, uranium oxide, in ores such as pitchblende. Refined uranium metal used in reactors is in the form of UO_2, uranium dioxide.

A sample of uranium was used by the French physicist Henri Bequerel in his discovery of the concept of radioactivity in 1896. Natural uranium has three predominant natural isotopes, ^{234}U, ^{235}U, and ^{238}U, all of which are radioactive; other uranium isotopes can be produced artificially in a reactor. The half lives of the natural isotopes are: 2.44×10^5 years for ^{234}U, 7.10×10^8 years for ^{235}U, and 4.5×10^9 years for ^{238}U; and their

composition in natural uranium by mass is: 0.005% ^{234}U, 0.711% ^{235}U, and 99.284% ^{238}U. Considering the isotope half lives and their mass percentages, it can be calculated that 48.9% of the radioactivity of natural uranium is derived from the isotope ^{234}U, 2.2% from ^{235}U, and 48.9% from ^{238}U [ATSDR 1999]. Thus, ^{234}U contributes as much to the radioactivity of natural uranium as does ^{238}U, despite the fact it is 20 thousand times less abundant. Natural uranium has a low specific radioactivity of about 0.68 µCi/g or 1.8 x 10^7 Bq, which means natural uranium is considered only a weakly radioactive element.

All isotopes of uranium, natural or manmade, decay by emission of alpha particles of various energies, a process by which the uranium is transformed into another element that is also radioactive. The decay series continues until reaching a non-radioactive isotope of lead. Alpha particles have very low penetrating power but deposit large amounts of energy during penetration. Thus, alpha particles represent little hazard when on the surface of the skin, but are potentially a significant hazard if inhaled or ingested, whereupon they come in close contact with sensitive tissues [Hartmann 2000]. Beta and gamma radiation are also emitted during certain transformations, but those radiation levels are lower. Workers exposed to natural uranium could receive radiation exposures to all of the isotopes in the transformation series.

The use of uranium as nuclear fuel or in nuclear weapons requires enrichment of the fissionable isotope ^{235}U. The enrichment process concentrates ^{235}U in the metal to specific activities required to sustain nuclear reactions. The by-product of enrichment is uranium with reduced levels of the ^{235}U isotope, or "depleted" uranium. The Nuclear Regulatory Commission considers the specific activity of DU to be no more than 0.36 µCi/g, but more aggressive enrichment processes can drive this value slightly lower (~0.33 µCi/g) [ATSDR 1999]. This means DU has roughly 50% of the radioactivity of natural uranium. Even though DU has less specific radioactivity than natural uranium, it retains all of its chemical properties. The large-scale production of enriched uranium for nuclear weapons and fuel over the decades has resulted in an abundance of cheap DU, a factor that has played a role in its use in a wide variety of applications (e.g., radiation shielding, compact counterweights, armor, kinetic energy weapons). The properties of DU that make it useful as an armor-penetrating munition are its density (1.68 times that of lead) and the ability to engineer into it a molecular structure that facilitates entry into a hardened target by "shedding" outer layers of the metal during penetration.

2.2 Uranium Toxicity and Health Effects

Toxicology studies of uranium relevant to understanding DU health effects are numerous, beginning with the first reported observations of uranium-induced kidney abnormalities in the middle 1800s (see [Hodge 1973b]). Most of our detailed knowledge of uranium toxicity is derived from studies in the 1940s and early 1950s as the Manhattan Project and the need for enriched uranium for reactors led to a requirement to understand better the occupational hazards presented to uranium workers. Much of that original work is described in the classic multi-volume monographs of Voeglen and Hodge [Voeglen 1949; Voeglen 1953) and in Hodge *et al.* [Hodge 1973a], which are often together considered the definitive compilations of toxicology and pharmacokinetic data for uranium in animals and humans.

Additional biological information about uranium has accumulated since then that has reinforced our understanding of both uranium and DU health effects. The Agency for Toxic Substances and Disease Registry (ATSDR) has produced a very thorough reference that summarizes what is known and not known about the toxic effects of uranium exposure [ATSDR 1999]. The controversy surrounding the use of DU during and after the 1991 Gulf War led to a number of other excellent literature reviews of uranium and DU health effects [Institute of Medicine 2000; The Royal Society 2001, 2002].

2.2.1 Cancer

2.2.1.1 Epidemiological Studies

A series of significant epidemiological studies of nuclear industry workers and uranium miners and millers carried out since the mid 1960s have added a wealth of data to the uranium health effects database. Several investigations of uranium millers [Wagoner 1965; Archer 1973; Waxweiler 1983], workers whose occupation exposes them to uranium dust inhalation in the workplace, used death certificates and in some cases health records to investigate cancers and other diseases (e.g., renal) as a cause of death. These studies failed to clearly identify a link between uranium exposure and any specific health effects, including cancer. Studies have also been carried out of workers at the Y-12 nuclear processing plant in Oak Ridge, Tennessee [Dupree 1995; Loomis 1996] The studies, which often included controls for age, race, gender, radiation dose, other chemical exposures, and medical history (when available) showed no association between cancer and occupational exposure to radiation from external and internal sources. The relatively small sizes of these epidemiological studies, uncertainties about the amount of uranium workers were exposed to, and the impact of confounding factors such as parallel exposures to agents such as radon, silicates, and other toxic metals (e.g., arsenic) lead to large statistical errors in the results, so caution should be exercised in over-interpreting the results of such studies.

In the 1991 Gulf War, an unknown number of personnel were exposed to DU aerosols (primarily uranium oxides) after being in vehicles that were struck by DU munitions, rescuing personnel in struck vehicles, reclaiming or investigating struck vehicles, or moving through areas where DU dusts were left in the environment. Even though satisfactory exposure model exists for such personnel, it is generally considered that the brief exposures to DU dust experienced by personnel would have been far below exposures experienced by uranium miners and millers in earlier studies, so no cancers would be expected by any route of exposure. McDiarmid *et al.* [McDiarmid 2004] calculated radiation dose estimates of personnel carrying DU shrapnel in their bodies as a result of fragment injuries. Whole body radiation counting using the ICRP 30 biokinetic model for uranium yielded an upper dose limit of 0.1 rem/year, a dose is not considered particularly dangerous.

2.2.1.2 Cancer in Laboratory Animals

Experiments with laboratory animals have expanded our understanding of the carcinogenic potential of uranium and DU. Not long after the 1991 Gulf War, in an effort to understand more about the potential health effects in personnel wounded in that conflict by DU shrapnel, Pellmar *et al.* [Pellmar 1999a] carried out a toxicological investigation using Sprague-Dawley rats implanted with various numbers of DU pellets (cylinders 1 mm in diameter and 2 mm long) to mimic shrapnel injuries in humans. Although cancer was not specifically designed as an endpoint in these studies, necropsies of subject rats showed no increased number of tumors in DU-implanted rats compared to tantalum pellet-implanted controls. The high levels of spontaneous tumor development typical of Sprague-Dawley rats confounded the interpretation of that data, however.

In similar studies, Hahn *et al.* [Hahn 2002] implanted male Wistar rats with either pellets or thin foils (1 × 2 or 2 × 5 mm) of DU. They showed that there was a slightly elevated risk of cancer in the DU foil- versus tantalum foil-implanted rats. Implanted DU pellets similar to those used in research by others [Pellmar 1999a] did not produce tumors. Interpretation of these data were complicated by the fact that rats are prone to developing tumors around implanted foreign bodies shaped like the foils used, irrespective of the object's chemical composition. In experiments assessing the carcinogenic potential of DU and a tungsten alloy proposed as a surrogate for DU in armor-penetrating munitions, Kalinich *et al.* [Kalinich 2005] demonstrated

DU pellets (1 x 2 mm cylinders) implanted into the leg muscles of Fisher 344 rats for 18 months caused no cancer development.

2.2.1.3 Uranium Genotoxicity

There have been very few studies comparing uranium exposure in humans to genotoxic endpoints. Such studies are relevant because destabilization of the genome can indicate an increased susceptibility to cancer development. Martin *et al.* [Martin 1991] reported that levels of chromosomal aberration, sister chromatid exchange, and dicentrics measured in nuclear fuel workers increase proportionally with uranium exposure. McDiarmid *et al.* [McDiarmid 2004], in their 10-year follow-up of thirty-nine veterans exposed to DU in friendly fire incidents during the 1991 Gulf war, reported that study participants exposed to the highest levels of DU showed a statistically significant increase in chromosomal aberrations compared to the low-exposure groups. HPRT mutation frequencies were also significantly higher in the high-DU groups, but sister chromatid exchanges were not.

Even though uranium appears to pose only a minor risk of cancer in both man and animals, the consistent observation of uranium-induced genetic changes remains a cause for concern since they are known to precede cancer development. Hu and Zhu [Hu 1990] injected uranyl fluoride into the testes of mice and showed that chromosomal aberrations in spermatogonia and primary spermatocytes are dependent on the amount of injected uranium. In experiments in which rats were implanted with pellets of DU and/or the biologically inert metal tantalum, urine and serum mutagenicity levels increased in a DU dose-dependent manner [Miller 1998a].

Experiments with cultured cells have also demonstrated the capacity of uranium to induce genotoxic changes. [Lin 1993] showed that uranyl nitrate increased frequencies of micronuclei, sister chromatid exchange, and chromosomal aberrations in Chinese Hamster Ovary (CHO) cells. Miller *et al.* [Miller 1998b] observed transformation of human osteoblast (HOS) cells to a tumorigenic phenotype after exposure to uranyl chloride. Treated cells demonstrated anchorage-independent growth, increased levels of the *k*-ras oncogene, decreased levels of Rb tumor suppressor protein, and an increase in sister chromatid exchange. Transformation rates were 9.6 times that of untreated controls, and transformed cells formed tumors in nude mice. Miller *et al.* [Miller 2002a] showed that incubating HOS cells with uranyl nitrate solutions at a fixed uranium concentration but increasing specific radioactivity resulted in increasing transformation rates and dicentrics. Results demonstrated that uranium toxicity results from both chemical and radiological toxicity. Miller *et al.* [Miller 2003] showed that uranium can activate gene expression through several signal transduction pathways that may be involved in the uranium toxicity and tumorigenicity.

The fact that human studies have yet to demonstrate a conclusive association between uranium exposure by any route and cancer [ATSDR 1999] is significant, but it should not discount the relevance of in vitro experiments showing genetic changes consistent with cancer development. The BEIR IV report [BIER IV 1988] on radon and other alpha particle emitters states that large statistical uncertainties in most of the epidemiological studies looking for cancer in uranium workers may be hiding small populations of adversely affected individuals; it cautions against minimizing the risk until more studies are available.

2.2.2 Nephrotoxicity

The review by Hodge [Hodge 1973b] of uranium toxicity prior to the Manhattan Project (1824-1942) shows that it has long been known that uranium is toxic to humans, animals, and other living things. Kidney toxicity of uranium was first recognized in animals around the middle of the 19th century, and kidney toxicity remains

the primary basis for the regulation of uranium exposure. Limits for inhalation and ingestion of uranium are aimed at not allowing uranium content in the kidney to exceed a set value, which for most countries is set at a maximum of 3 µg of uranium per gram of kidney tissue; effects caused by exposure of the kidneys at these levels are considered to be minor and transient.

The pharmacokinetics and pharmacodynamics of uranium and, therefore, DU are well established [Wrenn 1985; Leggett 1994; Taylor 1997; ICRP 1995; Leggett 2003]. There have been many studies that have investigated the results of uranium exposure in laboratory animals [Morrow 1982; La Touche 1987; Ortega 1989; Wren *et al.*, 1989]. Once absorbed it circulates in the blood primarily as the stable uranyl ion UO_2^{2+} bound to bicarbonate, albumin, or proteins [Diamond 1989; Kocher 1989; Leggett 1989]. At the kidney, uranium is filtered through the glomerulus and most is excreted within 24 h. Renal kidney toxicity occurs when residual uranium is subsequently taken up by the proximal tubules and causes damage by forming complexes with phosphate ligands and proteins in the tubular walls, thereby impairing kidney function [Blantz 1975].

Pellmar *et al.* [Pellmar 1999a] showed that DU from pellets implanted in muscle of rats can be measured in their urine within one day after pellet implantation. Over the course of the 18-month experiment, kidney uranium content reached levels well above 5 µg/g of kidney tissue, a concentration that, if reached in an acute exposure, would normally prove lethal to the animal. The findings suggested that the kidney adapted to the high levels during the chronic exposure.

2.2.3 Uranium and Bone

Bone is a major site of uranium deposition. Neuman and colleagues, in a series of early articles designed to understand how uranium interacts with normal bone metabolism, published the first observation demonstrating that bone has a high affinity for uranium. Twenty to thirty percent of a toxic does of intravenous uranium could be found in the bones of male rats within 2.5 hours after administration, and 90% of the uranium retained by the body after 40 days was in bone [Neuman 1948a]. They showed that young growing rats or rats deficient in dietary calcium incorporated greater amounts of uranium than controls [Neuman 1948b]. They also showed that uranium is preferentially incorporated in areas of active calcification and becomes more refractory to resorption as new calcification covers areas of uranium deposition [Neuman 1948c].

Uranium incorporates itself into the bone matrix by displacing calcium to form complexes with phosphate groups in the matrix [Domingo 1992; Guglielmotti 1989]. Bone-bound uranium establishes a equilibrium with uranium in the blood, and as the circulating uranium is excreted by the kidneys, bone-bound uranium slowly returns it to the circulation over time [Wrenn 1985]. Pellmar *et al.* [Pellmar 1999a] demonstrated that DU from implanted pellets rapidly distributes throughout the body and accumulates at high levels in the bone, though histological examination showed no bone lesions as a result.

2.2.4 Uranium Neurological Effects

The neurophysiological effects of uranium exposure have been investigated for many decades. Among the early findings was the observation that uranyl ions potentiate the twitch response of frog sartorious muscles by prolonging the active state of contraction. The fact this effect was reversed by administration of phosphate ions suggested that uranium prolongs the action potential [Sandow 1996]. In a study of uranium workers, Kathren and Moore [Kathren 1986] showed individuals excreting up to 200 µg U per liter urine manifested abnormal mental function. High doses of oral (210 mg/kg) or subcutaneous (10 mg/kg) uranyl acetate caused tremors in rats [Domingo 1987]. It was also shown that uranium applied at high concentrations to the ileal

longitudinal muscle of guinea-pig [Fu 1985] and mouse phrenic nerve-diaphragm preparation [Lin 1988] enhanced muscle contraction.

In a study investigating the toxicology of embedded pellets of DU in rats to mimic shrapnel wounds in wounded 1991 Gulf war veterans, Pellmar *et al.* [Pellmar 1999b] demonstrated that DU crosses the blood brain barrier and accumulates in the hippocampus, where electrophysiological changes were observed. Briner and Murray [Briner 2005] tested behavioral effects and brain lipid peroxidation in rats exposed to various concentrations of uranyl acetate in drinking water for 2 weeks or 6 months. Open-field behavior was altered in male rats receiving the highest dose of DU after two weeks of exposure; female rats demonstrated behavioral changes after 6 months of exposure. Lipid peroxidation levels increased in the brain and correlated with some of the behavioral changes, but the correlation was ambiguous. Barber *et al.* [Barber 2005] sought to determine the kinetics of uranium content in the brains of rats following a single intraperitoneal injection of uranyl acetate (1mg/kg). They found that uranium content in all areas of the brain tested increased rapidly after injection and remained elevated for 7 days post-injection. Interestingly, rats stressed by daily forced swimming before uranium injection accumulated less uranium in their brains and had lower levels than unstressed animals 7 days after exposure.

2.2.4 Uranium Reproductive/Developmental Effects

Despite nearly a century of studies of uranium toxicity, there were few detailed studies of uranium reproductive and developmental toxicity until the late 1980s [Domingo 1995]. In most exposure scenarios, including exposure to DU, the chemical toxic effects from uranium compounds appear to occur at lower exposure levels than radiological toxicity [Hartmann 2000], and this is thought to be the case for reproductive effects as well [Domingo 1995]. In the early 1980s, Domingo and his colleagues began extensive investigations of uranium reproductive toxicity, and they have provided most of our current knowledge on the subject to date. There are only a few and preliminary studies investigating the reproductive and developmental health effects of DU specifically. Given the likelihood that uranium chemical toxicity plays the major role in any reproductive and developmental toxicity, it is reasonable to assume that uranium and DU reproductive health effects are similar.

Early studies [Maynard 1949] identified uranium as a possible reproductive toxicant in rats. Male and female rats fed diets containing 2% uranyl nitrate hexahydrate for 7 months followed by normal diets for 5 months produced fewer litters with fewer pups per litter than control rats [Maynard 1949]. However, it was difficult to determine in these experiments whether uranium toxicity or nutritional effects arising from retarded weight gain in the uranium-fed rats caused the decreased reproductive success. In follow-up studies, rats fed diets containing 2% uranyl nitrate hexahydrate for a single 24-h period after weaning also produced fewer litters with fewer pups per litter than control rats with no signs of maternal toxicity [Maynard 1953], an observation that strengthened the connection between uranium exposure and reproductive toxicity.

Llobet *et al.* [Llobet 1991] showed that male mice continuously receiving water containing uranyl acetate dihydrate and mated with untreated females resulted in a significantly decreased, but dose-unrelated, pregnancy rate; but testicular function and spermatogenesis were unaffected. Domingo *et al.* [Domingo 1989] showed that pregnant female mice given uranyl acetate dihydrate (0.05-50 mg/kg per day) by oral gavage from gestational day 13 through postnatal day 21 demonstrated no significant decrease in litter size, pup litter size, and pup viability except at the highest dose (50 mg/kg; about 1/5 of the oral LD_{50}). On the other hand, injection of 1/40 to 1/10 the subcutaneous LD_{50} dose (20 mg/kg) into pregnant female mice between gestational days 6-15 produced both maternal and fetal toxicity [Bosque 1993]. Some of the malformations noted in pups could have occurred as a result of maternal toxicity, but defects such as cleft palate and certain

other variations are not known to be associated with maternal toxicity and were interpreted to be the result of uranium developmental toxicity [Domingo 2001].

Reports of the health status of military veterans the 1991 Gulf War have provided certain insight into the possible health effects of DU. A follow-up examination of DU-exposed individuals (via embedded DU fragments and/or inhaled DU dusts) showed that there were no significant differences in semen and sperm characteristics among veterans with high or low DU urine concentrations [McDiarmid 2000; McDiarmid 2004). As of 1999, fifty of the Gulf War veterans in the McDiarmid studies had fathered thirty-five children since the conflict, and none had birth defects [McDiarmid 2001]. The relatively small number of individuals involved in these studies and the end points that were possible limit their contribution towards understanding reproductive health effects.

2.3 Summary of Depleted Uranium Health Effects

Since internalization of uranium in any form will result in a combined chemical and radiation exposure, there exists the potential for subtle differences in health effects between DU and uranium. Recent developments in cell biology technology and understanding are providing more sensitive approaches towards understanding those differences (see [Miller 2001]). Although it is doubtful that future findings will alter the view that moderate exposures to either DU or uranium present a significant toxicological threat, the new information could help improve current risk assessments of DU exposure.

3.0 TUNGSTEN ALLOYS

3.1 Background

Tungsten metal has been used for many centuries in a variety of applications. Tungsten alloys are, as their name implies, primarily tungsten, and they can include a wide variety of other metals. The first effective use of tungsten in combat dates back to German and Allied munitions (mostly tungsten carbide) in World War II. Pure tungsten metal is hard, brittle, and very difficult to machine, but when it is mixed with various other metals, including nickel, iron, and cobalt, a variety of alloys can be produced, some of which have characteristics especially useful for military applications.

Based upon a small number of studies, prevailing theory is that elemental tungsten or insoluble tungsten compounds have only limited toxicity [Leggett 1997]. For example, tungsten coils implanted into the subclavian artery of rabbits rapidly degrade, leading to elevated serum tungsten levels as early as 15 minutes after implantation. However, after 4 months, no signs of local or systemic toxicity were observed [Peuster 2003].

Studies on health effects of nickel and cobalt are more numerous. Intramuscular injections (28 mg) of soluble metallic nickel or cobalt result in formation of rhabdomyosarcomas at the injection site. With nickel, 100% of injected rats develop a tumor within 41 weeks [Heath 1964], while administration of cobalt results in tumor formation in 40% of the rats with a latency period of 71 weeks [Heath 1954; Heath 1956]. However, intramuscular implantation of rods or pellets composed of various nickel or cobalt alloys used in orthopedic prosthetics results in no excessive tumor formation [Gaechter 1977; Sunderman 1989]. A variety of other nickel compounds, including nickel subsulfide, nickel oxide, and nickel monosulfide, have been tested for carcinogenic potential via intramuscular administration [Gilman 1962; Sunderman 1976; Sunderman 1977]. Tumors (rhabdomyosarcoma and fibrosarcoma) were found in many cases at the injection site, with tumor

yield dependent on solubility and concentration of the administered compound. It has been postulated that the yield of localized tumors is inversely related to the rate of solubilization of the nickel-containing compound [Kasprzak 1983]. This hypothesis does not appear to hold for cobalt compounds [Lison 2001].

Weinbren *et al.* [Weinbren 1978] reviewed the evidence that injected iron compounds of various kinds can cause local cancers in man. Only sporadic cancers of various types (mostly sarcomas) were discovered in the medical literature. The International Agency for Research on Cancer (IARC), focusing on a particular injectable iron compound, iron-dextran complex, determined that there is inadequate evidence for carcinogenicity in man [IARC 1987], but the evidence for carcinogenicity of iron dextran complex in animals suggested that it is reasonably anticipated to be a human carcinogen. Huang [Huang 2003] reviewed evidence of iron as a carcinogen and concluded that the influence iron can have on various oxidative mechanisms known to cause cancer suggests that iron can contribute to cancer development either as an cancer initiator or promoter. Until the mechanisms by which iron induces cancer are better understood, it remains uncertain whether iron or iron bearing compounds are carcinogenic.

When metals such these are alloyed, alloy-specific effects are observed that complicate understanding of the alloy's toxicity. Investigations of hard-metal disease have shown that either tungsten carbide or cobalt alone has limited toxicity on lung tissue [Lasfargues 1992]. However, when the metals are alloyed, the tungsten carbide/cobalt mixture increases the observed toxicity synergistically. It is not known whether this is due to the combined toxicity of the tungsten carbide/cobalt mixture or to an increase in the bioavailability of the known toxicant, cobalt [Lison 1997].

Studies performed by the Armed Forces Radiobiology Research Institute (AFRRI) with cultured human osteoblast sarcoma (HOS) cells, using tungsten, nickel, and cobalt or iron in proportions equivalent to the makeup of armor-penetrating munitions, demonstrated that the mixtures induce a metal dose-dependent malignant transformation [Miller 2000, 2001, 2002a; Kalinich 2005]. This neoplastic transformation was associated with genotoxic damage, including sister chromatid exchange, micronuclei induction, and DNA single-strand breaks [Miller 2000, 2001]. Data from these studies are summarized in Table 1. The data demonstrate that, like the studies of Lasfargues *et al.* [Lasfargues 1992], the metal combinations produce toxicity greater than the sum of the toxicities produced by the individual metals.

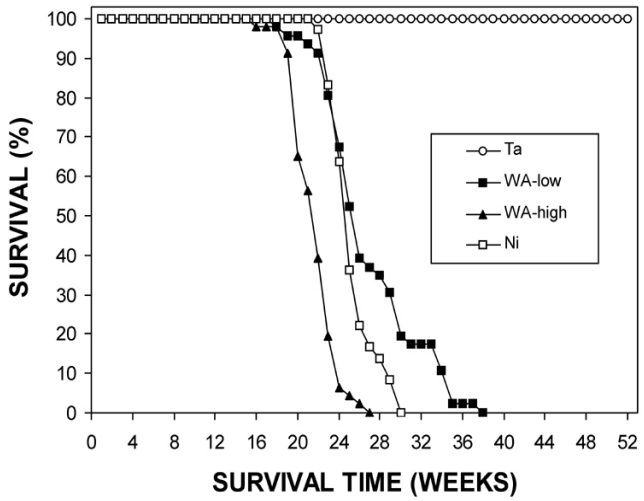
Table 1. Effect of tungsten alloy and alloy components on neoplastic transformation of HOS cells and tumorigenicity of those transformed HOS cells in athymic mice.

Treatment	Transformation Frequency of HOS Cells	Tumorigenicity (%) of Transformed HOS Cells
None	4.2	0
Co	4.8	0
Fe	5.1	0
Ni	9.5	0
W	6.9	0
W/Ni/Co	37.6	50
W/Ni/Fe	40.1	58

3.2 AFRRI Embedded Tungsten Alloy Studies in Rats

In 2001, AFRRI initiated a project funded by the United States Army Medical Research and Materiel Command (USAMRMC) to assess the carcinogenicity and immunotoxicity of DU and one of the tungsten-nickel-cobalt alloys of special interest to the military [Kalinich 2001]. In this study, rats received a low dose (4 pellets; cylinders 2 mm long and 1 mm in diameter) or a high dose (20 pellets) of the tungsten alloy implanted in the gastrocnemius muscles. The number of pellets used was within the range of doses that might be expected in wounded personnel, with 4 pellets representing about 2 ounces of shrapnel in man. Other rats received implants of the biologically inert metal tantalum as a control for pellet implantation. Results of this study [Kalinich 2005] showed that tumors developed in 100% of the tungsten alloy-implanted rats. Figure 1 shows that aggressive tumor development forced euthanasia of all of the alloy-implanted rats 37 weeks after implantation, with the higher dose of alloy forcing euthanasia as early as 16 weeks.

Figure 1. Survival times of pellet-implanted rats. Rats were implanted with either 20 pellets of W/Ni/Co tungsten alloy ("WA-high"), 4 pellets of tungsten alloy (plus 16 pellets of the biologically inert metal tantalum; "WA-low"), 20 pellets of tantalum ("Ta"), or 20 pellets of pure Nickel ("Ni"). Rats were sacrificed only when became moribund.



Gross examination of the tumors showed most to contain a tungsten alloy pellet embedded within it, while tantalum pellet-implanted rats showed no tumors (Figure 2). Tumors were malignant and characterized as extremely aggressive pleomorphic rhabdomyosarcomas (Figure 3). Furthermore, muscle tumor-derived metastases developed in the lungs of all alloy-implanted rats (Figure 4). No rats receiving tantalum pellets developed tumors. Interestingly, neither did the DU-implanted animals.

Figure 2. Effect of implanted W/Ni/Co pellets on F344 rats. (a) Gross appearance of Ta-implanted hind leg. (b) Dissected area around implanted Ta pellet (arrow indicates pellet). (c) Gross appearance of tungsten alloy-implanted hind leg with tumor(s). (d) Dissected area around implanted tungsten alloy pellet with tumor surrounding pellet (arrow indicates pellet).

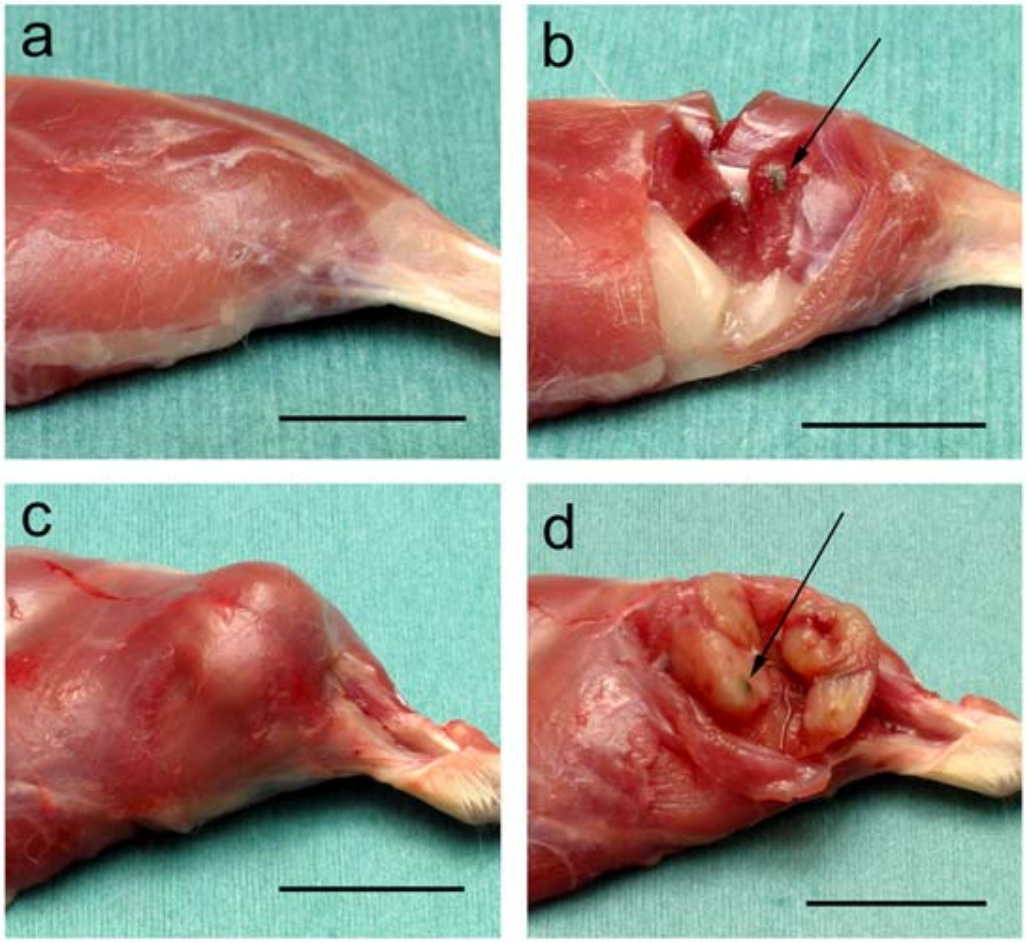


Figure 3. Histopathological examination of leg tumor surrounding W/Ni/Co pellet. (a) Hematoxylin and eosin (H&E) stained section of leg tumor from F344 rat showing tungsten alloy pellet hole (P), scale bar = 500 µm. (b) H&E stained tumor section showing neoplastic infiltration of preexisting muscle fibers (MF), scale bar = 200 µm. (c) H&E stained tumor section showing neoplastic cells with numerous mitoses (arrows) and bizarre mitotic figures (BZ), scale bar = 100 µm. (d) H&E stained tumor section showing pleomorphic cell (arrow), scale bar = 100 µm. (e) Desmin staining of leg tumor showing neoplastic cells (NC) and muscle fibers (MF), scale bar = 500 µm. (f) Desmin staining of neoplastic cells, scale bar = 50 µm.

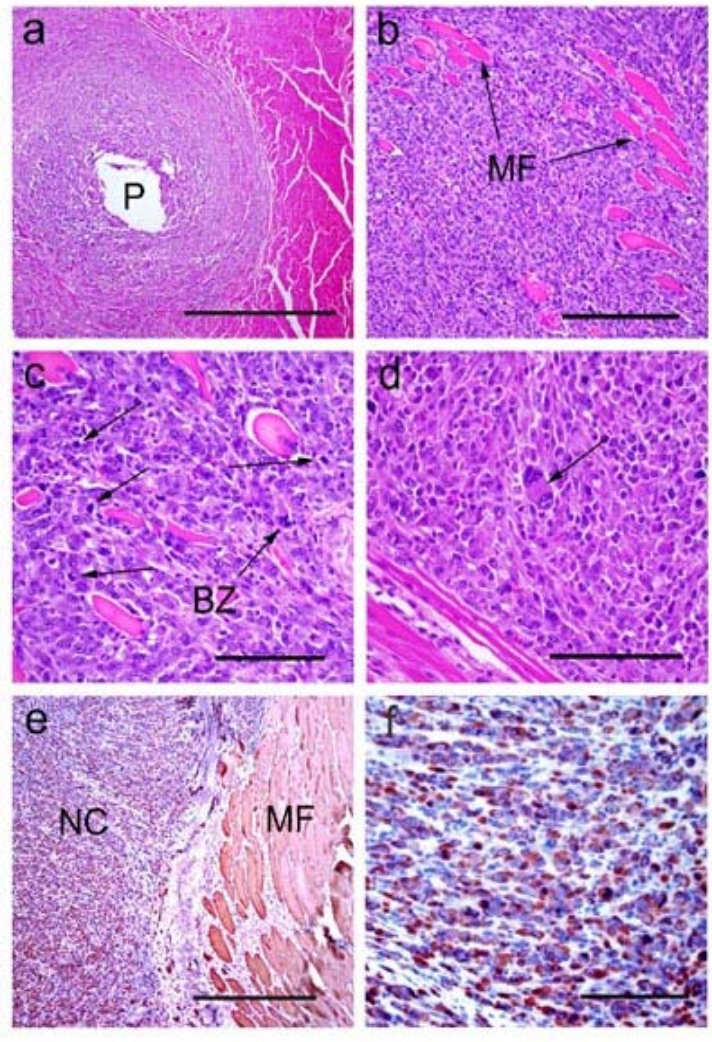
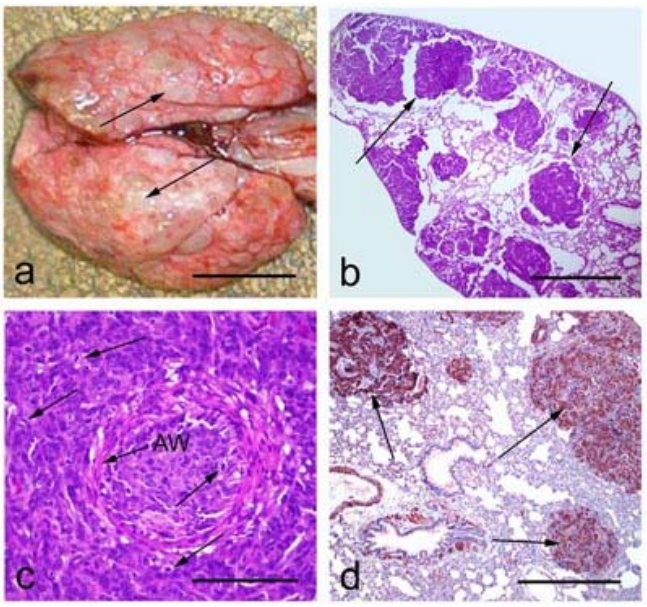


Figure 4. Lung metastases from W/Ni/Co-implanted F344 rats. (a) Gross appearance of pulmonary metastases from tungsten alloy-implanted rat (arrows indicate metastatic foci). (b) H&E stained section of pulmonary metastases (arrows), scale bar = 1 mm. (c) H&E stained section of an occluded pulmonary arteriole (arrow indicates vascular smooth muscle wall (AW)) showing neoplastic cells with numerous mitoses (arrows), scale bar = 50 µm. (d) Desmin staining of pulmonary metastases (arrows), scale bar = 500 µm.



The mechanism of tumor induction with embedded WA pellets remains unclear. The AFRRI study did not include a full assessment of the individual metals composing the tungsten-nickel-cobalt alloy, since funding was limited and the study focused on DU at a time when questions of tungsten alloy toxicity were only beginning to be raised [Miller 2000, 2001, 2002a]. However, it included experiments with one of the alloy component metals, nickel, a known carcinogen used as a positive control to validate the experimental system. The nickel–implanted rats (20 pellets) developed tumors, pleomorphic rhabdomyosarcomas, but the tumors developed significantly slower than those of the high-dose tungsten alloy groups (Figure 1; [Kalinich 2005]) and no lung metastases were observed. Nickel exposure in rats receiving the pure nickel pellets was presumably much higher than that in rats implanted with tungsten alloy pellets, which contained 6% nickel.

Despite the smooth and impermeable surface of the pellets, foreign body or solid-state carcinogenesis is unlikely because of the intramuscular, rather than subcutaneous, location of the implanted pellets [Bates 1966; Brand 1975]. In addition, implanted tantalum pellets of an identical geometry resulted in no tumor formation. One possibility is that free-radical reactions at the interface of the pellet and tissue could result in damage leading to carcinogenesis.

3.3 Summary of Tungsten Alloy Health Effects

For years, exposure to tungsten was thought to be of little consequence to health. In fact, tungsten is occasionally found as a minor component (5-15%) in some of the various alloys used to produce medical implant devices such as artificial hips and knees. Since the alloy used in WA munitions usually contains greater than 90% tungsten, along with smaller amounts of other metals, it was also assumed that exposure to these alloys would present little or no health risk.

As is shown here, this is not the case in the rat. Embedded WA pellets not only resulted in aggressive, metastatic, pleomorphic rhabdomyosarcomas, but also caused significant hematopoietic changes well before the carcinogenic effect was observed. It seems unlikely that these adverse health effects can be attributed solely to the small amounts of nickel and/or cobalt present in the alloy. Recent *in vitro* studies have demonstrated a synergistic effect in terms of damage when tungsten is present with these metals [Miller 2001, 2002a] that may play a role in WA carcinogenicity.

4.0 CONCLUSIONS

In many ways the development of substitutes for DU in munitions has followed a pattern similar to that for DU deployment, in that incomplete toxicological information was available prior to their release. In terms of the new tungsten alloys, it was assumed that many years of industrial use of tungsten and alloys such as tungsten carbide, which showed common exposures to the metals (e.g., inhalation, ingestion, or skin contact) represents a manageable risk, meant they could be used as safely in armaments. However, until recently, limited toxicological studies had ever been carried out with many of the alloys of specific military interest, and there has been no meaningful prior experience with exposures of special interest to the military, such as via embedded fragments. Recent research into the health effects of embedded pellets of a tungsten/nickel/cobalt alloy have led to those assumptions being questioned.

The health effects of tungsten metal exposure is receiving a new look in other circumstances as well. Environmental testing of the leukemia cluster around Fallon, Nevada, in the western United States showed slightly elevated levels of several heavy metals including uranium and cobalt, but significantly elevated levels of tungsten [CDC 2003]. Although no definitive link between elevated tungsten levels and cancer has been established, because of the uncertainty surrounding this issue, the U.S. National Toxicology Program recently added tungsten to their list of compounds to be assessed for adverse health effects. Further study of the health effect of tungsten and tungsten alloys is clearly indicated.

Our present understanding and experience reinforces the advisability of including more effective health effects testing early in weapons development programs. The relatively insignificant cost of such testing would be paid back many times over by helping to redirect expensive engineering programs to more acceptable alternatives earlier in the development process.

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